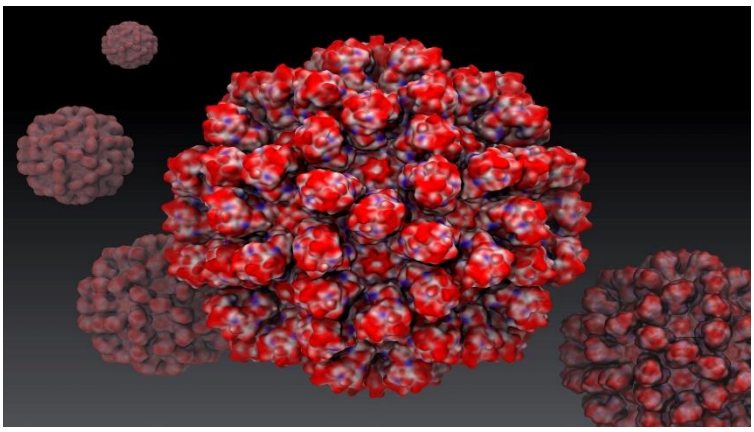

Genetic characterization of old and contemporary RHDV and RCV-like strains circulating in Portuguese rabbit populations

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2016

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Agradecimentos

Todo o trabalho desenvolvido nesta tese não seria possível sem o apoio de muitas pessoas, e como tal, gostaria de lhes agradecer por tudo.

À minha orientadora, Doutora Joana Abrantes, agradeço por toda a ajuda que me deu, desde o trabalho, a paciência e a disponibilidade para me ajudar em todos os passos desta tese, assim como todas as correções que fez.

Ao meu coorientador, Doutor Pedro Esteves, agradeço pela disponibilidade constante e por me ter aceite no grupo e ter possibilitado com que todo este trabalho acontecesse.

À comissão científica do Mestrado em Biodiversidade, Genética e Evolução, nas pessoas de Professor Doutor Paulo Alexandrino (em memória), do Professor Doutor António Múrias dos Santos e ao Professor Doutor Paulo Célio Alves, agradeço por me aceitarem neste mestrado e me fornecerem a formação essencial para a investigação científica.

Às “imídias”, agradeço por toda a ajuda que me deram, tanto no laboratório como nas correções da tese. Com a ajuda e apoio de todas as pessoas deste grupo, sei que o realizar desta tese se tornou muito mais fácil e agradável.

Queria também agradecer ao CIBIO, por me ter acolhido, e a todo o pessoal do CTM, pela disponibilidade de instalações e ajuda nas questões laboratoriais.

Agradeço a toda a minha família, em particular aos meus pais, que me ajudaram em todos os momentos da minha vida e me incentivaram a perseguir os meus sonhos, sem nunca me prenderem, e se sou a pessoa que sou hoje, é porque lhes devo tudo.

Aos meus amigos, mouros e espanhóis, agradeço por todos os momentos inesquecíveis que irei levar comigo para a vida. Sei que posso contar com todos vocês, e espero que estes fortes laços de amizade nunca se quebrem.

Finalmente, gostaria de te agradecer a ti, Nídia. Obrigado por quem és e pela pessoa melhor em que me tornas. Deste-me todo o apoio que sempre precisei, e sei que tudo isto não seria o mesmo sem ti.

Obrigado a todos, por tudo!

Summary

Rabbit haemorrhagic disease (RHD) is a fatal disease for European rabbits (*Oryctolagus cuniculus*) worldwide. The causative agent for RHD is the rabbit haemorrhagic disease virus (RHDV), a *Lagovirus* of the *Caliciviridae* family. RHDV is a positive-sense single-stranded ~7.4 kb RNA virus. Phylogenetic studies identified six pathogenic genogroups (G1-G6), as well as non-pathogenic related forms. In 2010, a new variant (RHDV2 or RHDVb) was detected in France, forming a new distinct phylogenetic group. This new variant differs from other pathogenic RHDV strains as it causes mortality in vaccinated adult rabbits, but also in young rabbits (<2 months old). In the Iberian Peninsula, this new variant replaced the former circulating strains (G1). Genomic characterization of RHDV2 from Portugal and Spain revealed multiple recombination events with G1 and non-pathogenic strains, suggesting the circulation of non-pathogenic forms in Iberia.

Recombination seems to play a key role in the evolution of the new variant. However, as it has also been detected sporadically in older strains, more information is required on the genomic composition and evolution of RHDV strains, in order to better understand the ecological and economic impacts of this virus in rabbit populations. Thus, this thesis focused on the full genomic characterization of RHDV2 strains circulating between 2014 and 2016 and G1 strains retrieved from samples collected in the mid-1990s, as well as on the detection of non-pathogenic strains circulating in Iberia.

For RHDV2 sequences, multiple recombination events were detected at two distinct breakpoints: the already described recombination breakpoint at the non-structural/structural junction; and a new event with a breakpoint located between the non-structural proteins p16 and p23. Phylogenetic analysis further confirmed the recombination events, where all capsid sequences belonged to RHDV2, while non-structural sequences belonged either to G1 or non-pathogenic lagoviruses. All recombinant p16 proteins seemed to belong to a distinct form of non-pathogenic lagoviruses. Thus, at least two types of non-pathogenic strains may circulate in Iberia.

Amino acid polymorphism analysis showed that p16 presents the highest diversity for RHDV2 recombinant strains. The selection analysis detected two putative positively selected codons at RHDV2 capsid protein: the newly detected codon 10, and codon 307, which had already been reported as being under selective pressure in classical and non-pathogenic strains.

The relationships between RHDV2 capsid sequences were depicted in a haplotype network in order to disclose possible geographical patterns of evolution. Furthermore, mean substitution rates for RHDV2 capsid sequences were estimated at 4.3×10^{-3} subs/site/year, which places the shared common ancestor at around 2007, only a few years prior to the first RHDV2 detection.

For archived rabbit samples, phylogenetic incongruences between the non-structural and structural coding regions for some of the sequences led to a more extensive genetic characterization. Recombination analysis revealed a recombination breakpoint at the non-structural/structural junction, which was further supported by the phylogenetic analysis. While the structural parts belonged to G1, the non-structural parts seemed to belong to a new genetic group. Analysis of genetic distances between this group and publicly available strains showed evidence that this may be a new genetic group that had not been previously detected.

Despite the evidence for non-pathogenic lagoviruses in Iberia, screening efforts did not detect any non-pathogenic strain. However, characterization of these strains may have great importance in understanding the evolution of RHDV and the emergence of pathogenicity. More efforts are needed in the characterization of full genome sequences of RHDV and non-pathogenic strains worldwide to fully assess the emergence, epidemiology and evolution of this highly impacting virus in rabbit populations around the world.

Keywords: RHDV; RHDV2; full genome; viral evolution; recombination; Iberian Peninsula; European rabbit.

Sumário

A doença hemorrágica viral (DHV) é uma doença fatal para o coelho-bravo (*Oryctolagus cuniculus*) em todo o mundo. O agente responsável pela DHV é o vírus da doença hemorrágica viral (VDHV), um *Lagovirus* da família *Caliciviridae*. O VDHV é um vírus de cadeia simples e de sentido positivo constituído por ARN e com um genoma de ~ 7.4 kb. Estudos filogenéticos identificaram seis genogrupos de estirpes patogénicas (G1-G6), assim como estirpes não-patogénicas. Em 2010, uma nova variante (VDHV2 ou VDHVb) foi detetada em França, constituindo um novo grupo filogenético. Esta nova variante difere das outras estirpes patogénicas do VDHV por causar mortalidade em coelhos adultos vacinados para as estirpes pertencentes aos genogrupos G1-G6 e também em coelhos jovens (com menos de 2 meses de idade). Na Península Ibérica, esta nova variante substituiu a estirpe G1 que aí circulava. A caracterização do genoma das estirpes de VDHV2 revelou a ocorrência de múltiplos eventos de recombinação com estirpes G1 e não-patogénicas, o que sugere que as estirpes não-patogénicas do vírus podem também estar presentes na Península Ibérica.

A recombinação parece desempenhar um papel importante na evolução da nova variante. No entanto, como este mecanismo foi também detetado em estirpes mais antigas, é necessária mais informação sobre a composição genómica e a evolução das estirpes de VDHV, de modo a melhor compreender o impacto ecológico e económico deste vírus nas populações de coelho-bravo. Assim, esta tese teve como objetivos a caracterização genética de estirpes VDHV2 que circularam na Península Ibérica entre 2014 e 2016 e de estirpes isoladas de amostras antigas, e a deteção de estirpes não-patogénicas que circulam na Península Ibérica.

A análise das sequências do VDHV2 permitiu detetar vários eventos de recombinação, em dois locais do genoma: o primeiro com um ponto de recombinação já descrito anteriormente e localizado na junção das proteínas não-estruturais e estruturais; e um outro evento com um novo ponto de recombinação localizado entre as proteínas não-estruturais p16 e p23. A ocorrência de recombinação foi confirmada pela análise filogenética, na qual todas as sequências obtidas agruparam com o VDHV2 para a cápside e VP10, enquanto as sequências não-estruturais agruparam com estirpes G1 ou não patogénicas. Todas as proteínas p16 onde ocorreu recombinação pertencem a um grupo distinto de lagovírus não-patogénico, sugerindo a existência de pelo menos dois tipos de estirpes não-patogénicas na Península Ibérica.

A análise dos polimorfismos aminoacídicos revelou que a proteína p16 possui a maior diversidade. A análise de assinaturas de seleção detetou dois codões possivelmente sob seleção positiva na cápside do VDHV2: o codão 10, que não tinha sido detetado antes em VDHV, e o codão 307, que já fora detetado como estando sob pressão seletiva noutras estirpes de VDHV e estirpes não patogénicas.

As relações haplotípicas entre as sequências da cápside do VDHV2 foram representadas numa rede de haplótipos, para visualizar um possível padrão geográfico de evolução. A taxa média de substituição calculada para cápsides de VDHV2 foi de 4,3 x 10^{-3} substituições por sítio por ano, sugerindo a existência de um ancestral comum para esta estirpe por volta de 2007, apenas alguns anos antes da primeira deteção de VDHV2.

Relativamente às sequências de VDHV obtidas das amostras antigas, as incongruências filogenéticas entre as partes não-estruturais e estruturais de algumas amostras levaram a uma caracterização genética mais aprofundada. A análise de recombinação revelou um sítio de recombinação na junção dos fragmentos não-estruturais/estruturais, que foi confirmado posteriormente por análise filogenética. As partes estruturais agruparam com estirpes G1, enquanto as partes não-estruturais pareceram formar um novo grupo genético. O cálculo das distâncias genéticas entre este novo grupo e as estirpes disponíveis em bases de dados públicas revelou que este pode ser um novo grupo genético que não foi previamente detetado.

Apesar das evidências de circulação de lagovírus não-patogénicos na Península Ibérica, não foram detetadas quaisquer estirpes não-patogénicas no nosso estudo. No entanto, a caracterização destas estirpes pode ter grande importância na compreensão da evolução do VDHV e na aquisição de patogenicidade. Assim, são necessários maiores esforços na caracterização de genomas completos do VDHV e das estirpes não-patogénicas para compreender melhor o aparecimento, a epidemiologia e a evolução deste vírus nas populações de coelho-bravo.

Palavras-chave: VDHV; VDHV2; genomas completos; recombinação; evolução viral; Península Ibérica; coelho-bravo.

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Abbreviations

BEB: Bayes Empirical Bayes

bp: base pairs

cDNA: complementary deoxyribonucleic acid

DIC: disseminated intravascular coagulation

EBHSV: European brown hare syndrome virus

FEL: Fixed Effects Likelihood

FUBAR: Fast Unconstrained Bayesian Approximation

GTR+G+I: general time-reversible + gamma distribution + invariable sites

HPD: highest posterior density intervals

kb: kilobase pairs

LRT: likelihood ratio test

MCC: maximum clade credibility

MCMC: Markov chain Monte Carlo

MEME: Mixed Effects Model of Episodic Diversifying Selection

ML: Maximum Likelihood

MRCV: Michigan rabbit calicivirus

mya: million years ago

NGS: Next Generation Sequencing

NNI: Nearest-Neighbor-Interchange

ORF: open reading frame

P: protrusion

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

qRT-PCR: quantitative real-time polymerase chain reaction

RCV: rabbit calicivirus

RdRp: RNA-dependent RNA polymerase

RHD: rabbit haemorrhagic disease

RHDV: rabbit haemorrhagic disease virus

RNA: ribonucleic acid

RT-PCR: reverse transcriptase polymerase chain reaction

S: shell

SLAC: Single Likelihood Ancestor Counting

TA: annealing temperature

tMRCA: time to the most recent common ancestor

UTR: untranslated region

VPg: viral protein genome-linked

1. Introduction

1.1. Rabbit haemorrhagic disease

Rabbit haemorrhagic disease (RHD) is a highly lethal and contagious disease occurring in domestic and wild European rabbits (*Oryctolagus cuniculus*) [1] and affects both rabbit subspecies, *O. c. cuniculus* and *O. c. algirus* [2]. The first case of this viral disease was reported in 1984 in the Jiangsu Province of the People's Republic of China [3], where an outbreak occurred among Angora rabbits commercially imported from Germany. This led to the death of 140 million domestic rabbits in less than one year [3,4]. The disease was then reported in Italy in 1986 [5] and quickly spread throughout Europe [1]. In the Iberian Peninsula, RHD was first reported in Spain in 1988 [6], followed by Portugal in 1989 [7], and drastically reduced wild rabbit populations [8]. In the following years, severe RHD outbreaks were also detected in North Africa and South America [9], as the aetiological pathogen quickly spread globally, mostly due to commercial trading [10]. As RHD became endemic across the world, naturally occurring outbreaks began to be observed worldwide [1].

Mortality rates due to RHD are very high, ranging between 70-90%, but in naïve populations mortality may reach 100% [1]. However, RHD does not affect young rabbits (<2 months old), presumably due to maternal inherited passive immunity [11,12]. Susceptibility increases gradually up until 8 to 9 weeks, when rabbits become fully susceptible [13]. Different immune responses are triggered following infection that account for the different course of the disease in young and adult rabbits. Indeed, while young rabbits develop a rapid and adequate inflammatory response in the liver, with a small fraction of hepatocytes being infected, accompanied by an increase in both T and B cells that allows them to resist the pathogen [14], such response is not observed in adult rabbits as they die too quickly to build up an effective response [12,15]. Nonetheless, young rabbits may act as agents for long-term disease spread, as persistent high values of liver transaminases suggest chronic infections [12]. The importance of an adequate immune response is further confirmed by the loss of resistance to RHD in immunosuppressed young rabbits [16].

RHD incubation period varies from one to three days and rabbits usually succumb within 12 to 36 h after the onset of fever. According to its clinical evolution, the disease may be classified as peracute, acute, subacute and chronic [1]. In the peracute form, animals suddenly die without exhibiting any signs of clinical infection. In acute infections,

rabbits present signs of neurological disturbance (e.g. convulsions, ataxia, paralysis, opisthotonos) and respiratory distress (dyspnea and epistaxis) as well as anorexia, apathy, dullness and side recumbency, followed by increased heart and respiratory rates prior to death [17]. Haemorrhages may be detected in the eyes (lacrimation), the mouth and/or the snout (mucohaemorrhagic nasal discharge), along with signs of hematuria and bloody diarrhoea [17,18]. The subacute course presents similar but milder symptoms, and rabbits usually survive by acquiring antibodies that confer resistance to a posterior infection [19]. Finally, during an RHD outbreak, a small part of the rabbit population may experience a chronic evolution of the infection, presenting severe and generalized jaundice, weight loss and lethargy. Although these animals may die in the following weeks, most likely due to hepatic dysfunction, those that survive develop high titres of antibodies that confer resistance up to one year [2,19].

RHD induces acute hepatic necrosis that can lead to disseminated intravascular coagulation (DIC), hepatic encephalopathy and nephrosis. Upon necropsy, the liver appears pale and swollen, with a distinctive lobular pattern. The spleen is depleted of lymphocytes and, along with the kidneys, also appears swollen and dark, due to the DIC [17,20]. The lungs may appear congested or haemorrhagic, and the trachea presents hyperaemic mucosa, in many cases also presenting a foamy liquid. The viscera and subcutaneous tissues may present ecchymosis and petechial haemorrhage, with intravascular coagulation causing thrombi in blood vessels [17,20].

Infected rabbits develop leukopenia (decreases in the abundance of both heterophils and lymphocytes), which tends to progress up to the death of the animal, and an increase in liver enzymes (serum alanine amino transferase, aspartate amino transferase, lactate dehydrogenase, alkaline phosphatase and γ-glutamyltransferase) is also observed. This overproduction of liver enzymes is responsible for changes in sugar and lipid metabolism, leading to hyperlipidemia and hypoglycemia [15,21].

The main transmission route of the disease is the oral-faecal, but other direct and indirect routes are possible [1]. Indirect routes include mechanical transmission via insects as flies feeding on animal carcasses that have died from RHD are efficient vectors and allow for long-distance dispersal or fomites [1,22].

The impact of RHD on natural populations is dependent on several factors, including climate variables, the interaction with other rabbit infections (e.g. myxomatosis), rabbit population dynamics and the carrying capacity of the habitats [23,24]. In wild rabbit populations, vaccination has not proven efficient in containing the disease [23,25,26]. On the contrary, outbreaks in rabbitries are mainly controlled resorting to vaccination programs [1]. Vaccines are produced either with inactivated virus

extracted from liver suspension of rabbits infected with RHD or with myxoma virus vaccines expressing RHD virus capsid protein [25,26]. Up to date no effective treatment for RHD exists, although there are some experimental studies with encouraging results on reducing liver damage and increasing survival rates [27,28].

1.2. Etiological agent

RHD is caused by rabbit haemorrhagic disease virus (RHDV), which along with the European brown hare syndrome virus (EBHSV) form the genus *Lagovirus* of the family *Caliciviridae*. This family also includes the genera *Nebovirus*, *Sapovirus*, *Vesivirus* and *Norovirus* [29]. Similarly to other caliciviruses, RHDV virions are small (35-40 nm of diameter) non-enveloped particles, arranged in a T = 3 icosahedral symmetry, and present the cup-shaped depression characteristic of all caliciviruses (Figure 1a) [1].

RHDV is a polyadenylated positive-sense single-stranded RNA virus of ~7400 nucleotides, organised into two open reading frames (ORFs) that overlap by 17 nucleotides [30]. ORF1 comprises nucleotides 10-7044 and encodes a large polyprotein (c.a. 257 kDa) that is cleaved by a trypsin-like cysteine protease, producing the non-structural proteins p16, p23, helicase, p29, viral genome-linked protein (VPg), protease and RNA-dependent RNA polymerase (RdRp), as well as the major structural protein VP60. ORF2 comprises nucleotides 7025-7378 and codes for the minor structural protein VP10 (c.a. 10 kDa) (Figure 1b). Additionally, the structural proteins are also encoded by a subgenomic RNA present in viral particles [1]. Both subgenomic and genomic RNAs have the VPg attached to the 5' end. It has been suggested that VPg, as well as the 5' untranslated region (UTR), may play a key role in viral replication and translation [31]. The function of the non-structural proteins p16, p23 and p29 still remains to be fully assessed. The protease is responsible for the auto-cleavage of the polyprotein, while the helicase and RdRp are associated with virus replication [32-34]. More recently, RdRp has also been associated with the recruitment of membranes of the secretory pathway and the establishment of correct replication complexes [35].

The capsid structure comprises 180 VP60 proteins organised into 90 dimeric capsomers. Each VP60 protein is composed of an N-terminal arm, a shell (S) domain, which protects the viral RNA from the environment, and a protrusion (P) domain, further subdivided into the subdomains P1, which constitutes the bottom of the protrusion, and P2, which forms the top [36]. The S domain is conserved among caliciviruses, while the P1 sub-domain is only moderately conserved and the P2 sub-domain presents high

sequence variability, most likely due to being more exposed to the host immune response and due to its role on binding to host cell receptors [37].

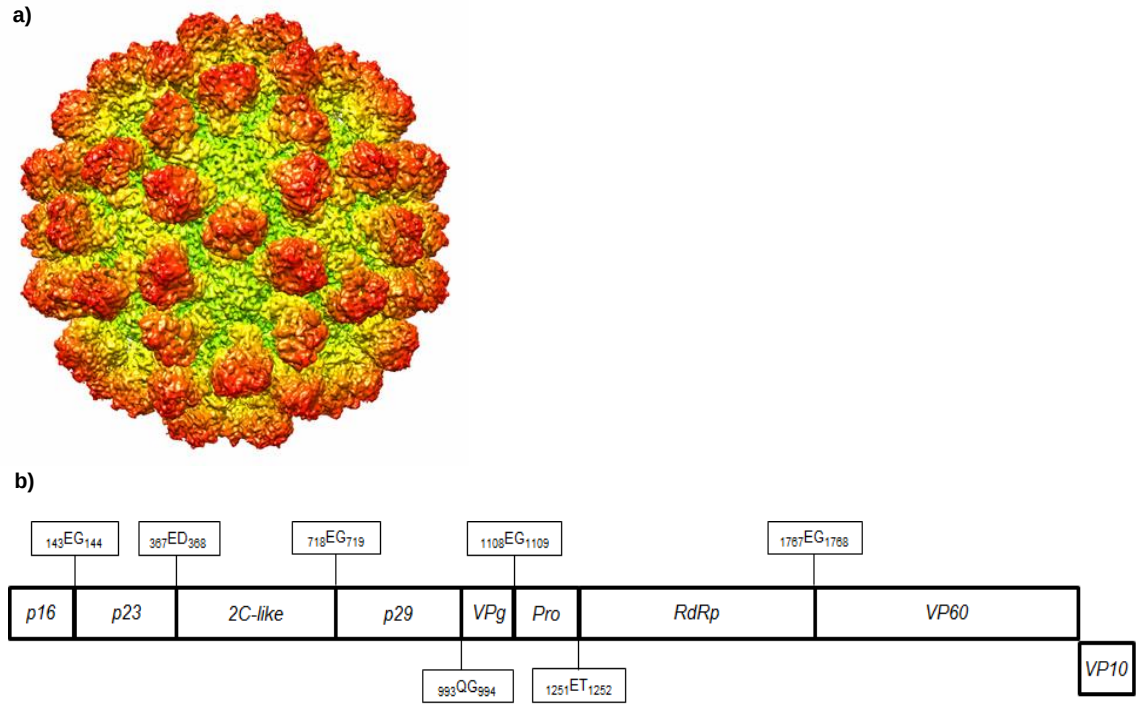


Fig. 1. RHDV virion particle and genomic organization. **a)** Reconstructed cryoEM map of the RHDV virion (adapted from [35]). **b)** RHDV genomic organization, with the expected cleavage sites and the respective coding proteins (adapted from [1]).

The inability to replicate RHDV in a cell culture system hinders the study of several aspects of the virus, including the life cycle [1]. However, the reverse transcriptase polymerase chain reaction (RT-PCR) has allowed for an extensive assessment of RHDV phylogenetic relationships. Phylogenetic studies on French RHDV samples identified several genogroups, consistent with a temporal rather than a geographical distribution, with the successive replacement of viral strains [38]. Strains were divided into six genogroups (G1-G6), with G6 representing the first antigenic variant of RHDV [2]; more recent studies suggest that G3, G4 and G5 belong to a single group (Figure 2) [39]. The antigenic variant G6, also referred to as RHDVa, is mostly associated with the rabbit production industry, and despite having different antigenicity, there is cross-protection between G6 and other strains [40,41]. Although several authors have used only partial

VP60 sequences to assess RHDV evolution [38,39,42], it has become evident that only the full sequence of the capsid gene allows for a robust and unequivocal phylogenetic inference [43-45].

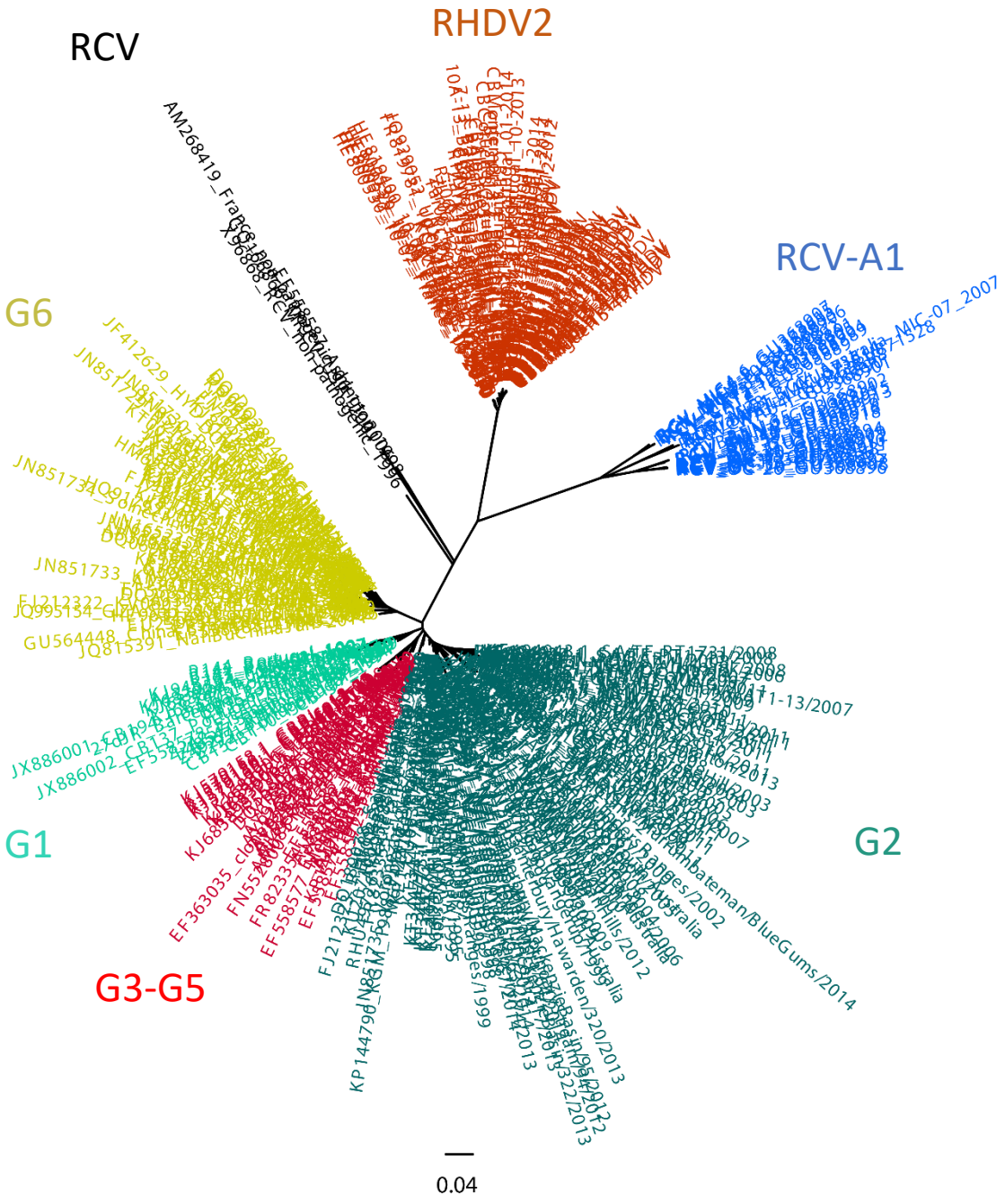


Fig. 2. Phylogenetic relationships between pathogenic and non-pathogenic RHDV strains. Colours represent different genetic groups. The tree was obtained using complete sequences of the capsid gene representative of RHDV diversity. The maximum likelihood method was used with 500 bootstrap replicates and the GTR+G+I model.

In the Iberian Peninsula, and until 2011, only G1 strains were found in wild rabbit populations [45-47], following the temporal pattern of evolution identified in France [48]. RHDV was also described in the Portuguese archipelago of Azores in late 1980s, and the characterization of the circulating strains showed an independent evolution of G3-G5-like strains in these islands, suggesting a non-Iberian origin [47,49].

Serological studies undertaken on asymptomatic rabbit populations detected anti-RHDV antibodies, which allowed to foresee the existence of RHDV-like non-pathogenic strains [50,51]. The first non-pathogenic strain (referred to as rabbit calicivirus, RCV) was identified in Italy in 1996 [52], and other strains were later identified both in Europe [53,54] and in Australia (designated RCV-A1; Figure 2) [55]. A moderately pathogenic strain, MRCV, was also characterized in the USA, although there are discrepancies in its phylogenetic and virulence assessment [44,56,57]. In the Iberian Peninsula, there is also evidence for the existence of RCV-like strains, as a recent study on currently circulating strains revealed recombination events outside of the capsid with strains being phylogenetically close to these non-pathogenic [43], but the capsid of these strains remains to be identified and characterized. Cross-protection conferred by non-pathogenic strains seems to vary, ranging from no protection to full protection [52,53,58], and it appears to be dependent on the time span between infections rather than on the titres of the cross-reacting antibodies [59].

Emergence of pathogenicity in lagoviruses is not yet fully understood. The most accepted hypotheses are either through a species jump or evolution from pre-existing non-pathogenic strains [60]. The first hypothesis is consistent with massive introductions of the American leporid *Sylvilagus floridanus* into Europe and emergence of RHDV and EBHSV at around the same time [60]. The other hypothesis is supported by the detection of anti-RHDV antibodies in rabbit samples collected prior to the first documented RHDV outbreaks [13] and the characterization of several non-pathogenic strains [52-55]. These hypotheses, however, remain to be tested. Interestingly, an association has been made between virulence and mutations in the non-structural protein p16 [61], although its biological role is still undetermined. Furthermore, other mutations have also been related to host-cell binding, virus entry and viral replication that might have had an influence in the acquisition of virulence [61].

1.3. A new RHDV variant

In 2010, a new variant of RHDV was discovered in France [62]. This new variant was responsible for severe outbreaks in both rabbitries and wild rabbit populations, registering mortalities up to 80-90% [62], resembling those observed in the early RHDV outbreaks. The new variant, referred to as RHDV2 or RHDVb, managed to rapidly spread throughout Europe, including the Iberian Peninsula [63-68]. Not long after, RHDV2 was also detected in the Azores and Australia [69,70], and more recently in the Canary Islands [71]. Sequencing of RHDV2 capsid gene revealed a new phylogenetic group with 80-82% identity with other lagoviruses, falling between European and Australian non-pathogenic strains (Figure 2) [62].

RHDV2 is antigenically different from G1-G6 [62]. Genome analysis showed that the majority of nucleotide differences are located in the P domain of VP60, in which the main antigenic regions are located [64,72]. Furthermore, sites under positive selection for the classical RHDV were not detected in RHDV2, but a new site was identified [73], suggesting the existence of distinct epitopes [64]. This was confirmed by the existence of differences in the recognition patterns of several monoclonal antibodies between classical RHDV strains and RHDV2 [64,68].

In addition, and in contrast to G1-G6, RHDV2 inflicts mortality in both vaccinated (vaccines that protect against G1-G6 strains) and non-vaccinated adult and young rabbits [62]. Experimental infections revealed that rabbits experience a longer course of the disease, presenting lower but more variable mortality rates and a higher number of subacute/chronic cases [63,64]. Moreover, RHDV2 is able to infect also *Lepus corsicanus, L. capensis* and *L. europaeus* where no forms of RHDV were ever detected, suggesting that it may have a broader host range [68,74,75]. Nonetheless, the species barrier was probably breached due to spillover events derived from severe RHDV2 outbreaks, in habitats shared between *Oryctolagus* and *Lepus* where exposure and infection pressure are high [75].

Despite its recent emergence, RHDV2 appears to be replacing circulating strains [64,73,76,77], most likely due to a selective advantage. Although the mechanisms responsible for such replacement are yet unknown, multiple recombination events in RHDV2 genomes involving G1 and non-pathogenic strains have been reported in Iberian Peninsula [43]. A major recombination breakpoint is located at the 5' of VP60 and splits the genome into two regions: one that encodes the structural proteins VP60 and VP10, which originates from RHDV2; and one that encodes the non-structural proteins, that may be either from RHDV2, G1 or non-pathogenic strains [43]. Thus, recombination may

play an important role in the evolution of this new variant and such events should be taken into consideration in future studies.

1.4. Mechanisms of evolution of RHDV

The origin and evolution of viruses remain difficult to trace mainly due to the high mutation rates observed and the large viral population sizes [78]. This is particularly important for RNA viruses, that can exhibit mutation rates in the order of one mutation per genome per round of replication [79] due to the lack of proof-reading and post-replicative error correction mechanisms [80].

Different genome variation mechanisms are explored by viruses, including mutation, recombination and reassortment [81]. Although some mutations may be deleterious, most are silent. In other cases, mutations that confer increased fitness in their host environment might appear and become fixed in the viral population [82]. The high number of viral replicates per generation further assures the production of many viable virus particles [78].

The rate at which mutations accumulate over time in an evolving virus lineage is designated as substitution rate and depends both on the mutation rate and natural selection [83]. There have been numerous attempts to estimate RHDV substitution rates and the time to the most recent common ancestor (tMRCA). Results vary, with estimates ranging between 5.5×10^{-4} and 4.2×10^{-3} nucleotide substitutions per site per year, and consequently tMRCA ranging from several decades to a few years prior to the first known case of RHDV [10,46,84]. Recently, a similar study revealed that by removing inaccurately dated sequences from the analysis, RHDV emergence could be traced back to late 1970s/early 80s, shortly before RHD emergence in China [85].

Likewise, a study on the Australian non-pathogenic strains (RCV-A1) showed that they may have arrived ~150 years ago alongside the introduction of rabbits in the continent [86]. However, a recent study showed that non-pathogenic lagoviruses exhibit similar or even higher evolutionary rates than pathogenic strains, and time estimates revealed that they were likely introduced into Australia along with introduction of myxomatosis as a biocontrol agent in 1950 [44].

Recombination also plays a key role in viral diversity and evolution. Recombination corresponds to the formation of a chimeric virus with genomic segments from different parental origins (Figure 3) [87]. This mechanism has been associated with expansion of viral host range, increases in virulence, evasion of host immunity and resistance to antiviral drugs, thus being responsible for major changes during viral adaptation [87].

The most common type of recombination, and the most likely to produce viable molecules, is the replicative (copy-choice) mechanism [88]. For this to occur, viral strains must co-circulate in the same geographical area during the same period of time and co-infect the same host cell [89]. The formation of viable recombinants is highly dependent on the genetic homology of strains. If the recombinant strain presents an adaptive advantage over the existing strains, either by mixing viable genotypes or by eliminating deleterious mutations, it may become the predominant strain due to natural selection [87].

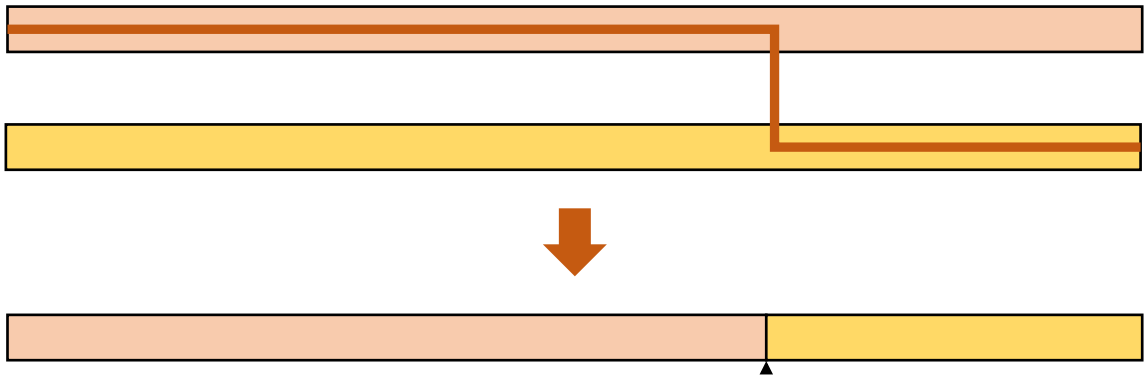


Fig. 3. Recombination scheme. Two different strains recombine to form a new chimeric strain. Arrowhead indicates the recombination breakpoint

For lagoviruses, recombination breakpoints have been described within the capsid and non-structural proteins [42,44,90,91] and between the non-structural and the structural part of the virus [43,44]. The high number of recombinant strains between the non-structural and the structural part of the virus reported for RHDV2 and recently also described for RCV-A1 suggests that recombination may play a major role in the new variant's emergence and/or evolution [43,44]. Furthermore, recombination may also facilitate cross-species transmission, as it enables changes in large portions of the genome and increases the likelihood of forming genetic configurations that allow host invasion [87]. Interestingly, RHDV2 has indeed managed to cross the species barrier and infect several *Lepus* species [68,74,75].

As any other virus, RHDV is part of the natural system and influences host genetic diversity, by a dynamic process of co-evolution. Likewise, the host impacts virus evolution, since it evolves towards developing resistance to pathogens, and consequently the virus will evolve to escape the host natural defences. This never ending cycle will deeply impact natural host populations dynamics and survival [92].

1.5. The European rabbit: the RHDV natural host species

The European rabbit, *Oryctolagus cuniculus*, is a member of the Leporidae family (that includes rabbits and hares), which along with Ochotonidae (pikas) constitutes the widely spread order Lagomorpha [93]. The oldest fossil record known in Lagomorpha, dating back to the early Eocene (~53 million years ago, mya), was found in India, which is in accordance with the accepted origin of lagomorphs in Asia [94].

Ochotonidae and Leporidae are differentiated by their number of teeth (26 and 28, respectively) and by ear and tail length [93]. Ochotonids have only one extant genus, *Ochotona*, with 30 species distributed over Asia and North America [95]. On the other hand, leporids are distributed over 11 genera with 62 extant species, inhabiting large areas in Europe, Asia, Africa and North and Central America. The genus with the highest number of species is *Lepus*, with 32 species, followed by *Sylvilagus* (17 species), *Pronolagus* (4 species) and *Nesolagus* (2 species). The remaining genera (*Brachylagus, Bunolagus, Caprolagus, Oryctolagus, Pentalagus, Poelagus* and *Romerolagus*) are monotypic [95].

Oryctolagus' fossil data suggests an Iberian origin for modern-day *O. cuniculus* [96]. During the Quaternary glaciations, two main refugia, a larger one located in the southwest of Iberia, and a smaller one located in the eastern Mediterranean coast, led to the differentiation of two rabbit subspecies at ~1.8 mya, the *O. c. algirus* in the southwest and the *O. c. cuniculus* in the eastern coast [97,98].

A higher genetic diversity is observed in *O. c. algirus* (that differentiated in the larger refugium) [97]. *O. c. cuniculus* expanded from northern Spain to France, suffering a bottleneck due to the geographical barrier of Pyrenees, and which resulted in a loss of genetic diversity [99,100]. Even though both species are genetically differentiated, they share a contact zone from the southeast to the northwest of Iberia where hybridization might occur (Figure 4) [101]. The origin of rabbit domestication, attributed to French monks circa 1400 years ago, started with a few number of *O. c. cuniculus* individuals. Gene flow between the subspecies due to secondary contact predating domestication rendered *O. c. algirus* as an indirect contributor for the genetic pool of the domestic breeds [102].

In Europe, and particularly in the Iberian Peninsula, rabbits are key species in the ecosystem. Several predators rely on rabbits as their main source of food, including the vulnerable Spanish imperial eagle (*Aquila adalberti*) and the endangered Iberian lynx

(*Lynx pardinus*) [103,104]. Indeed, reintroduction efforts of the Iberian lynx in Iberia are severely dependent on rabbit abundancy [105].

RHD is an important contributor to the main decrease on rabbit numbers, although hunting and habitat loss and fragmentation also played a role in population decline even prior to RHD emergence [8,106]. Aside from the ecological impacts, rabbit population decline has severe economic impact in the fur and meat industry, as well as in the hunting industry, as rabbits are important game species in Iberia [107].

Fig. 4. *O. c. algirus* (OCA) and *O. c. cuniculus* (OCC) distribution in the Iberian Peninsula. The dark grey area represents the contact zone between both subspecies.

In contrast, in the Australian continent, rabbits are seen as biological pests. After their introduction in the 19th century for hunting purposes, rabbits rapidly dispersed throughout the island and were responsible for the decline and even extinction of native species due to shelter and resources competition [108]. Rabbits were also considered agricultural pests, as they modified the Australian habitat [109,110]. In an effort to contain rabbit population expansion, myxoma virus, and then later RHDV, were introduced as biocontrol agents [111]. Although initially RHDV had the expected impact, rabbit populations seem to have developed resistance, thus reverting the initial success of RHDV as a pest control [112,113].

1.6. Objectives

This work focused on collecting information about past and current circulating lagovirus strains in Portuguese rabbits. Special attention was given to the new variant RHDV2 in order to assess its evolution, resorting to phylogenetic reconstruction and to the detection of recombination and signatures of selection, to better understand the mechanisms underlying its sudden emergence and fast establishment.

Thus, the main objectives of this thesis were:

- Full genome amplification and characterization of RHDV2 strains circulating in Portuguese wild rabbit populations between 2014 and 2016;
- Gathering insight on the evolution of RHDV2 in Portugal by performing phylogenetic reconstruction, identifying signatures of recombination and selection, detecting phylogeographic patterns and estimating substitution rates and time to the most recent common ancestor (tMRCA);
- Full genome amplification and genetic characterization of old circulating RHDV strains;
- Development of a screening methodology for the identification of non-pathogenic lagovirus strains circulating in Portugal;
- Sequencing and characterization of complete genome sequences of non-pathogenic lagovirus strains.

2. Methodology

2.1. Characterization of RHDV2 evolution in Portugal

2.1.1. Sample collection – survey “SOS Coelho"

Samples used in this study were collected from rabbits found dead in the field, as part of a surveillance program under the project “SOS Coelho” implemented in Portugal to monitor RHD between 2014 and 2016. No live animals were trapped, shot or handled to obtain tissues, thus no animal ethics’ permit was necessary. Animals were submitted to necropsy and samples of blood, liver, heart, lung, spleen and duodenum were collected and stored at -20 ºC for future analysis. Prior to freezing, duodenum samples were thoroughly washed with a phosphate-buffered saline (PBS) solution (see Annex I for the duodenum collection protocol) in order to remove organic material that could interfere with RNA extraction.

2.1.2. RNA extraction and cDNA synthesis

Up to 30 mg of liver were homogenized in a rotor-stator homogenizer (Mixer Mill MM400, Retsch) at 30 Hz for seven minutes. Total RNA extraction was performed with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers’ instructions.

RNA was quantified in a Nanodrop 2000 (Thermo Scientific) and 1-5 µg of RNA were used for reverse transcription. This was performed with the GRS cDNA synthesis kit (GRISP, Porto, Portugal) using oligo(dT) as primers, according to the instructions provided in the manufacturers’ protocol.

2.1.3. Lagovirus detection and genome amplification

In order to detect the presence of lagoviruses, PCRs were performed with two pairs of primers. Firstly, a broader pair of primers that allows for the detection of G1 and RHDV2 was used: RHDV4831F (5’ GTGTATGCCATGACTCCGAT 3’) and

EBHSV_VP60_0467R (5’ GCGTCGATGACAACATGAG 3’), with the forward primer located upstream of the capsid gene and the reverse primer located within the capsid gene. Secondly, PCR amplification was performed with a pair of primers specific to detect the new RHDV variant: RHDV6186F (5' CATTGACCACGACAGAGGTAAC 3') and RHDV6748R (5' CGTTAGTTGAACCGGCCTCAG 3'), both located within the capsid gene (see Annex II for PCR conditions).

From the samples that revealed to be positive for RHDV2, 19 were selected for complete genome sequencing. Samples were chosen according to their year and place of collection and to their genomic composition [43] (see Annex III for sample information). The complete genome sequence of each sample was obtained with a genome walking strategy, by amplifying several overlapping fragments ranging from ~200 to ~1700 bp, and using an array of primer pair combinations that cover the entire coding sequence. This process was optimized according to each type of strain [43] (see Annex III).

PCR reactions consisted of 0.6 – 1 µl of cDNA, 2 pmol of each primer, 5 µl of Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, Waltham, MA, USA) and water up to a final volume of 10 µl. PCR products were tested on a 2% agarose gel electrophoresis; samples with correct band size were then purified by adding 1 µl of ExoSAP to the PCR reaction and submitting the sample to 15 minutes at 37 ºC followed by 15 minutes at 80 ºC for enzyme inactivation. After purification, a sequencing reaction was performed with 1 µl of the purified product, 0.6 µl of the amplification primer, 0.5 µl of TRR, 1 µl of TRR buffer and water up to a final volume of 10 µl, with the following conditions: 3 minutes at 94 ºC and 25 cycles of 10 seconds at 96 ºC, 5 seconds at 50-58 ºC and 4 minutes at 60 ºC. Sequencing was completed on an automatic sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). Blast searches were performed in http://blast.ncbi.nlm.nih.gov/Blast.cgi for sequence confirmation and genogroup assessment; sequences were then aligned using ClustalW [114] implemented in BioEdit version 7.0.9.0 [115].

2.1.4. Data analysis

2.1.4.1. Recombination analysis

Nineteen complete coding sequences of RHDV2 strains obtained in this study, excluding the 5’ and 3’ UTRs, were screened for recombination using the RDP software [116]. The alignment also included non-recombinant samples representative of all RHDV

major groups (G1-G6, RHDV2 and non-pathogenic lagoviruses; n=90 sequences, 7368 nucleotides; see Annex IV for GenBank accession numbers). The following parameters were used: sequences were set to linear, Bonferroni correction, highest acceptable *P* value of 0.05 and 100 permutations. Eight methods (RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, 3Seq and LARD) were employed, and only breakpoints detected by three or more methods were considered.

2.1.4.2. Phylogenetic analysis

Phylogenetic analysis of the 19 sequences was carried out for each genome partition defined according to the recombination breakpoints. One breakpoint was located at the RdRp/VP60 boundary, splitting the genome into non-structural (nucleotide positions 10-5296) and structural (nucleotide positions 5305-7368) proteins. Additionally, for eight RHDV2 samples, another recombination breakpoint was detected between p16 and the remaining non-structural proteins, such that phylogenetic analysis was carried out also for the p16 protein. All phylogenetic trees were constructed with the Maximum Likelihood (ML) method available in MEGA7 [117], using the GTR+G+I model of nucleotide substitution as determined in MEGA7 with five discrete gamma categories and employing a Nearest-Neighbor-Interchange (NNI) heuristic method. The support for each node was determined from 500 bootstrap replicate ML trees. The analysis was conducted with the same dataset used for the recombination analysis (n=90 sequences; 7368 nucleotides; see Annex IV).

2.1.4.3. Amino acid polymorphisms

The alignment of the nucleotide sequences obtained for RHDV2 was translated to amino acids using MEGA7 [117]. Polymorphism diversity was measured for each protein according to their assigned genogroup and RDP results. Thus, analysis of the non-structural proteins of the obtained sequences was performed by comparison with RHDV2 strains and with older strains according to their genomic composition (G1 and non-pathogenic; see Annex IV for dataset partitions). Polymorphisms for the structural protein VP60 were compared with publicly available sequences of RHDV2 (n=158 sequences; 1737 nucleotides; see Annex IV). The diversity of each viral protein was calculated as the percentage of differences taking into account the length of the protein.

2.1.4.4. Selection analysis

Detection of signatures of selection was conducted for VP60 sequences obtained in this study and all publicly available RHDV2 capsid protein sequences (n=158 sequences; 1737 nucleotides; see Annex IV). Under neutrality, the ratio of non-synonymous (d_N) to synonymous (d_S) substitutions per site (ω) is expected to be one. Statistically significant deviations from this value can be evidence of positive ($\omega>1$) or negative ($\omega<1$) selection. Codon-specific positive selection was detected with several methods available in Datamonkey web server of HyPhy package v2.4 [118]: Single Likelihood Ancestor Counting (SLAC), Fixed-Effects Likelihood (FEL), Mixed Effects Model of Episodic Diversifying Selection (MEME) and Fast Unconstrained Bayesian Approximation (FUBAR).

Additionally, the analysis was carried out by comparing the fit to the data of two nested site specific models implemented in PAML4 [119]: the first pair includes M1a (nearly neutral) and M2a (selection) while the second pair comprises M7 (neutral, beta) and M8 (selection, beta+ω). Models M1a and M7 correspond to the null hypothesis and models M2a and M8 are the alternative hypothesis that allows positive selection. Then, a likelihood ratio test (LRT, which evaluates the difference between twice the log likelihood values of the paired models) was compared against a X^2 table in order to determine whether the data fit better a selection model than a neutral model. If significant differences existed, then the Bayes Empirical Bayes (BEB, P>95%) method was used to identify the codons under positive selection.

Only sites detected by three or more methods with P values <0.05 and/or with posterior probability values >0.95 were considered as being under selection.

2.1.4.5. Haplotype network

A haplotype network was constructed in order to identify a possible geographical pattern of evolution for the new RHDV variant. The RHDV2 capsid dataset previously used for the selection analysis (n=158 sequences; 1737 nucleotides; see Annex IV) was analysed in DnaSP [120]. A Median-Joining network [121] was constructed using the Phylogenetic Network software [122] with default options.

2.1.4.6. Substitution rate and tMRCA estimation

For this analysis, information of the sampling year was attributed for each sequence in the RHDV2 capsid dataset (n=158 sequences; 1737 nucleotides; see Annex IV). Only capsid sequences were chosen as recombination events outside of the capsid may interfere with results. The GTR+G+I substitution model was determined in jModelTest [123] as the best to model fit the dataset. Evolutionary rates were estimated as the number of substitutions per site per year (subs/site/year) using the Bayesian Markov chain Monte Carlo (MCMC) method available in the BEAST software [124], followed by the estimation of the tMRCA. The growth model that more accurately fits the dataset was determined as the exponential growth coalescent model with a lognormal relaxed clock. BEAST analysis was performed for 200,000,000 generations until convergence was achieved, with a sampling frequency of 20,000. To verify the reliability of the run and establish an adequate burn-in, Tracer v1.6 [125] was used. A burn-in of 1,000 trees (10%) was chosen. Finally, a maximum clade credibility (MCC) tree was created with TreeAnnotator v1.8 [126], with mean node heights and Bayesian posterior probability values reflecting the degree of support for each node.

2.2. Characterization of old RHDV samples

2.2.1. Sample collection, RNA extraction and cDNA synthesis

Recombination occurs frequently in RHDV2 [43], but for classical RHDV few examples are described [42,90,91]. In order to search for recombination in archived samples, we screened livers of rabbits found dead in the field and in rabbitries, collected as part of a surveillance program implemented in Portugal to monitor RHD between 1993 and 2000. Tissue samples were stored at -20 ˚C.

RNA extraction and cDNA synthesis were performed as previously described (section 1.2.).

2.2.2. PCR amplification

A previous scanning of the collected samples retrieved 95 positive samples for lagoviruses (Lopes et al., unpublished observations). Blast searches of these sequences allowed the determination of their genogroup, showing that 91 of these samples had the highest similarity with G1. However, four of these samples, P16, P19, P30 and P95 (see Annex V for sample information), presented inconclusive results as for the fragment upstream of the capsid the same highest identity (~87%) was observed with strains belonging to different genogroups (JF438967_G1, KP144792_BLA1994_G2, EF558576_Jena_G3-G5). For the partial sequence of the capsid, the highest identity (~97%) was found with G1 strains (e.g. Z29514_SD, EF558578_Eisenhuttenstadt). In order to clarify this pattern, the complete genome was amplified for these four samples following the previously described genome walking strategy (section 2.1.3.), with the same PCR and sequencing conditions.

2.2.3. Data analysis

2.2.3.1. Recombination analysis

The full genome of old recombinant samples sequenced in this study, excluding the 5' and 3' UTRs were screened for recombination using the RDP software [116]. The dataset included non-recombinant samples representative of all RHDV major groups (G1-G6, RHDV2 and non-pathogenic lagoviruses; n=83 sequences; 7368 nucleotides; see Annex VI for GenBank accession numbers). Recombination analysis parameters were set as described in section 2.1.4.1.

2.2.3.2. Phylogenetic analysis

Phylogenetic analysis of obtained sequences was carried out using the same dataset as for the recombination analysis (n=83 sequences; 7368 nucleotides; see Annex VI), with genome partitions according to the detected recombination breakpoint located at the RdRp/VP60 boundary, splitting the genome into non-structural (nucleotide

positions 10-5296) and structural (nucleotide positions 5296-7368) proteins. Phylogenetic analysis parameters were set as described in 2.1.4.2.

2.2.3.3. Genetic distances

Between groups mean distances were calculated in MEGA7 between the group composed of old strains sequenced in this study (P16, P19, P30 and P95) and the major genetic groups: G1, G2, G3-G5, G6, RHDV2 and non-pathogenic lagoviruses (n=83 sequences; 7368 nucleotides; see Annex VI for genetic group assignment). Distances were calculated for the genome partitions defined after the recombination analysis, with 500 bootstrap replicates, p-distance and partial deletion for gaps/missing data treatment (95% site coverage cutoff).

2.3. Identification and characterization of non-pathogenic strains in Iberian Peninsula

2.3.1. Sample collection

Samples collected under the “SOS Coelho” project that revealed to be negative for the presence of pathogenic lagoviruses were selected for the screening of non-pathogenic lagoviruses. The latter present a different tissue tropism and are mostly found in the rabbit duodenum [55], thus duodenum samples of both European rabbits and Iberian hares (*L. granatensis*) were used for the screening. All duodenum samples were washed again to further remove remnants of organic material. RNA was extracted from a total of 187 samples (see Annex VII for sample information) and cDNA was synthesized as described above.

2.3.2. PCR screening

The strategy for detecting non-pathogenic strains consisted in a PCR screening with two pairs of primers: a universal pair for lagoviruses detection, located within the

capsid gene, Rab1b (5' CAGCDSGCACTGCYACCACAGCATC 3') and Rab2 (5' GAAKCKRAACTGCATGCCACCAGCCCA 3') [55]; and a second pair of primers that amplifies a fragment of the non-structural protein p16, RHDV0078F (5' CTTCCTGGACCTCAGGGACAAG 3') and RHDV0270R (5' CATGTGGGTCCGAATTTGTGC 3') (see PCR conditions in Annex II). PCR reactions consisted of 0.6 – 1 µl of cDNA, 2 pmol of each primer, 5 µl of Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, Waltham, MA, USA) and water up to a final volume of 10 µl. PCR products were tested on a 2% agarose gel electrophoresis.

Optimization of PCR conditions with the universal primer pair Rab1b and Rab2 included G1-G6 and RCV-A1 samples (RCV-A1 samples were kindly provided by Dr. Tanja Strive from CSIRO, Australia), and the closely related EBHSV. For the second primer pair, which was designed to be specific for non-pathogenic lagoviruses, optimization was achieved with non-pathogenic/RHDV2 recombinant strains, as these strains have the non-structural backbone characteristic of non-pathogenic strains.

However, with these two pairs of primers no conclusive results were obtained. Thus, all samples were submitted to another round of screening, relying on quantitative real-time PCR (qRT-PCR). This method allows for a more sensitive and robust screening [127].

2.3.3. qRT-PCR

In order to determine the best suited pair of primers for the qRT-PCR, traditional PCRs were performed in two samples previously amplified in our laboratory that presented non-pathogenic non-structural proteins [43], along with an RCV-A1 sample. From the primers tested, the pair RHDV0078F + RHDV0270R showed to specifically amplify the three samples, thus being the chosen pair for the qRT-PCR screening.

The optimal temperature for the qRT-PCR reaction with this pair of primers was then determined with a temperature gradient. The three non-pathogenic representative samples were used on the qRT-PCR reaction and tested for temperatures ranging from 50-64 ºC. The optimum temperature chosen was 62 ºC, as it was the highest temperature with the lowest Ct (cycle threshold, defined as the number of cycles required for the fluorescence signal to cross the threshold) [127].

The qRT-PCR was performed in a CFX-96 Real-Time PCR Detection System (Biorad, California, USA) and amplification of a 192 bp fragment (positions 78 to 270) was carried out using 0.4 µM of each primer, 5 µl of iTaq Universal SYBR Green

Supermix (Biorad, California, USA), 1 µl of cDNA and water to a final volume of 10 µl. Cycle conditions used were 30 seconds at 95 ºC followed by 40 repeats of a two-step cycle with 5 seconds at 95 ºC and 30 seconds at 62 ºC. A melting curve was obtained through a final ramping cycle from 65 ºC to 95 ºC with intervals of 0.5 ºC.

3. Results and discussion

3.1. Characterization of RHDV2 evolution in Portugal

3.1.1. Recombination and phylogenetic analysis

The complete coding sequence of 19 RHDV2 strains circulating in Portugal between 2014 and 2016 was determined (see Annex III for sample information). Recently it was shown that recombination occurs frequently in RHDV2 strains from the Iberian Peninsula [43]. Thus, in order to account for this and better understand the full extent of recombination in these samples, the aligned sequences were screened using RDP [116]. Seven methods confirmed 13 sequences as recombinants with high statistical support (P values <0.001; Table 1), with a consistent recombination breakpoint located near the boundary between the non-structural and the structural part (nucleotide positions 5234-5399). RHDV2 was identified as the donor for the structural backbone of all samples, while for the non-structural part, parental strains belonged to either G1 or non-pathogenic lagoviruses closely related to the Australian calicivirus RCV-A1 (GenBank accession number EU871528) (Table 1). This recombination event is consistent with that described for other RHDV2 Iberian strains [43].

Additionally, for 8 samples, RDP detected another recombination event (P values <0.05; Table 1), with a breakpoint located near the p16/p23 boundary (nucleotide positions 355-470). The most likely parental donor for the p16 of these recombinant strains is the strain CBAnd1 (GenBank accession number KP090976). Thus, in these cases, a second recombination event occurred in the non-structural part, between a non-pathogenic lagovirus distinct from RCV-A1 and either G1 or RHDV2 (Table 1). Accordingly, these samples presented a deletion of codon 68 only observed for CBAnd1 [43].

Taking into account the RDP results, ML phylogenetic trees were constructed for the structural and non-structural parts (excluding p16). As expected, all samples clustered in a highly supported RHDV2 genogroup for the structural proteins (bootstrap value of 100; Figure 5a). As for the non-structural part, sequences fell into three genetic groups: six samples clustered with recent RHDV2 strains; eight samples clustered with previously circulating G1 strains; and five samples grouped with non-pathogenic strains similar to the Australian calicivirus (bootstrap values ≥90; Figure 5b).

Table 1. Results of the recombination analysis for RHDV2 samples using RDP.

Strains	Most likely donor strain			Genogroup			Breakpoint †	Methods (average P value)						
	p16	NS*	S**	p16	NS*	S**		RDP	GENECONV	BootScan	MaxChi	Chimaera	SIScan	3Seq
SOS89 SOS125 SOS129 SOS133 SOS158 SOS474	CB194		CBVal16	G1		RHDV2	5254-5345	4.53E-114	3.99E-102	2.09E-104	1,72E-34	3.58E-32	1.46E-47	1.46E-116
SOS137 SOS164	CBAnd1	CB194	CBVal16	NP2‡	G1	RHDV2	355-394	1.45E-16	2.57E-03	3.71E-17	2.06E-07	9.79E-08	3.59E-02	-
							5234-5329	6.26E-93	3.26E-91	2.71E-93	3.21E-34	9.31E-35	3.03E-46	2.62E-108
SOS148 SOS149 SOS151 SOS173 SOS404 SOS468	CBAnd1	Seg08-12		NP2‡	RHDV2		446-470	6.02E-32	1.03E-16	5.17E-24	1.81E-10	1.12E-09	-	1.09E-14
SOS140 SOS150 SOS155 SOS473 SOS492	RCV-A1		Zar11-11	NPǂ		RHDV2	5248-5307	1.24E-105	1.03E-112	6.90E-117	1.61E-31	3.83E-29	1.34E-50	7.80E-04

*Non-structural proteins
**Structural proteins
†95% confidence interval
ǂNon-pathogenic strains similar to RCV-A1
‡Non-pathogenic strains similar to CBAnd1

For the eight samples detected as recombinants for the p16/p23 boundary, a ML tree that considered only the p16 coding region was constructed. This confirmed that these samples possess a p16 protein that fell closer with non-pathogenic lagoviruses. More interestingly, they form a highly supported cluster (bootstrap value of 96; Figure 5c) with isolate CBAnd1, which appears in a separate group from other non-pathogenic strains collected in Iberia.

These results show that, in addition to the detected non-pathogenic lagovirus/RHDV2 (Figure 6a) and G1/RHDV2 (Figure 6b) recombinants at the non-structural/structural junction that had already been reported in Iberia [43], there are two additional types of recombinants circulating in Portugal: the combination of non-pathogenic lagoviruses closely related to CBAnd1 for the p16 with RHDV2 coding sequences for the rest of the genome (NP2/RHDV2 recombinant; Figure 6c); and the occurrence of triple recombinants, constituted by the p16 sequence also closely related to CBAnd1, a G1 backbone for the remaining non-structural proteins and RHDV2 as the donor for the structural proteins VP60 and VP10 (NP2/G1/RHDV2 recombinant; Figure 6d). Strikingly, despite this distinct non-pathogenic like strain has now been detected in Portuguese rabbit samples collected in 2015, it had only been detected once from a Spanish rabbit sample collected in 2012, suggesting that this strain still circulates and has recombined with RHDV2, at least for the p16 protein.

The biological implications of such recombination pattern remain to be addressed and although the role of p16 is not yet well defined, it has been suggested that mutations in this protein may be related with increased virulence [61]. It is thus tempting to speculate that these samples may have different virulence when comparing to other RHDV2 strains with no recombinant p16. As all samples were collected from dead animals with lesions compatible with RHDV, it is safe to assume that, like recombinant strains presenting a non-structural subset belonging to non-pathogenic lagoviruses, these strains are still pathogenic.

Interestingly, no non-recombinant RHDV2 strains were detected. All sequences that were thought to be non-recombinant in our first screening had the recombination event at the p16/p23 boundary. This may indicate that these new recombinants possess an advantage over non-recombinant RHDV2 strains, possibly at optimal levels of virulence. Thus, it would be of major interest to perform experimental infections to assess the functional relevance of the new recombination events reported here and their impact on virulence.

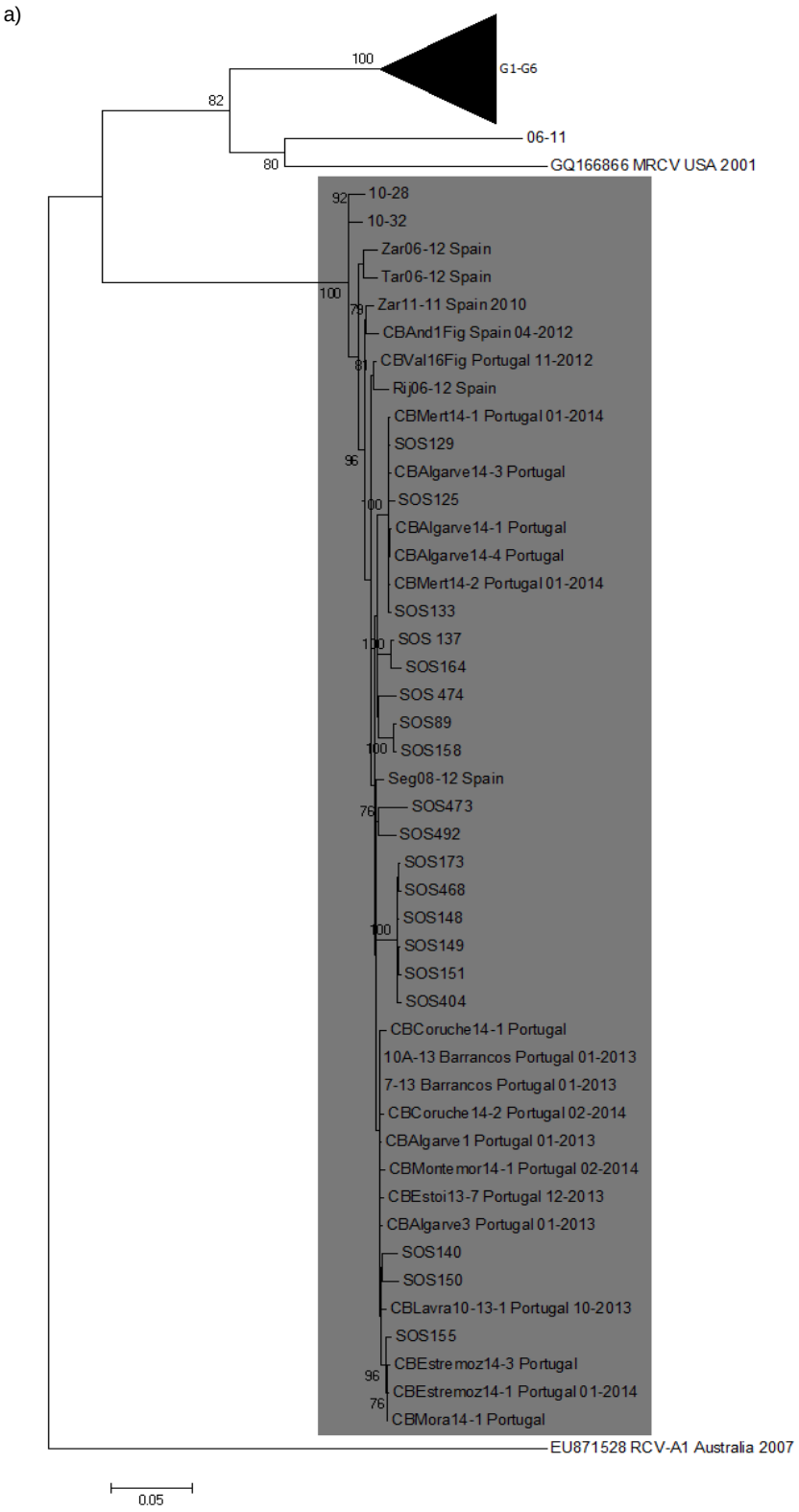
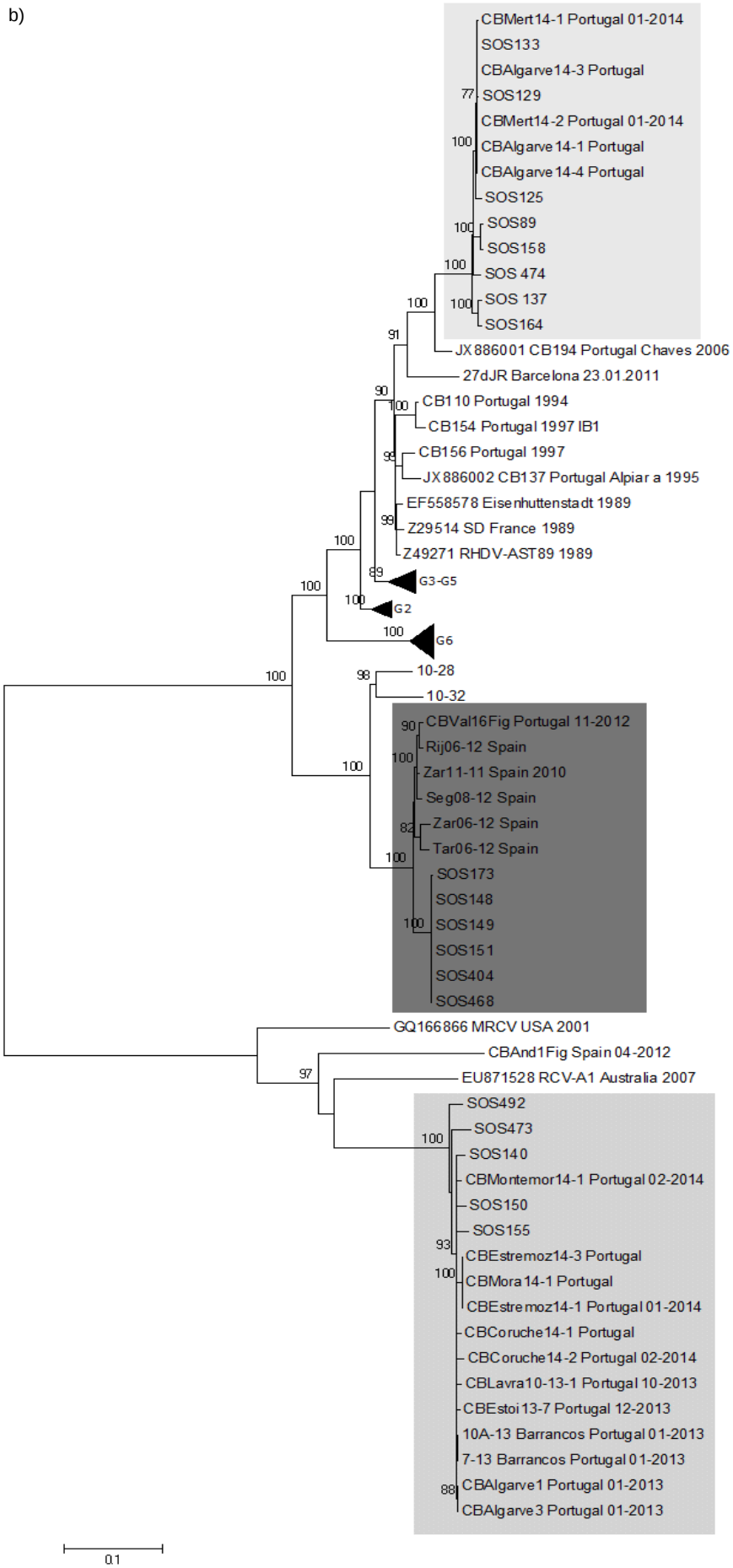


Fig. 5. ML phylogenetic trees for genome regions defined by the recombination analysis in RHDV2 samples. Genogroups containing the sequenced strains are shaded. The remaining genogroups are collapsed and indicated. Horizontal branch lengths are drawn to a scale of nucleotide substitutions per site and the tree is mid-point rooted. Only bootstrap values higher than 74 are shown for key nodes. a) ML tree based on the capsid sequence including VP60 + VP10 (nucleotides 5296-7368). RHDV2 sequences are shaded dark grey. b) ML tree based on non-structural protein sequences (excluding p16) (nucleotides 430-5295). Sequenced strains fall into three distinct genogroups: G1 (shaded light grey, non-pathogenic lagoviruses (medium grey) and RHDV2 (dark grey). c) ML tree based on p16 (nucleotides 1-429). Sequences fall into G1 (shaded light grey) or into two distinct non-pathogenic groups, related to either RCV-A1 (medium grey) or CBAnd1 (dark grey).

b)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SOS133
CBAlgarve14-3 Portugal
SOS129
CBMert14-2 Portugal 01-2014
CBAlgarve14-1 Portugal
CBAlgarve14-4 Portugal
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SOS 137
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JX886001 CB194 Portugal Chaves 2006
27dJR Barcelona 23.01.2011
CB110 Portugal 1994
CB154 Portugal 1997 IB1
CB156 Portugal 1997
JX886002 CB137 Portugal Alpiar a 1995
EF558578 Eisenhuttenstadt 1989
Z29514 SD France 1989
Z49271 RHDV-AST89 1989
G3-G5
G2
G6
10-28
10-32
CBVal16Fig Portugal 11-2012
Rij06-12 Spain
Zar11-11 Spain 2010
Seg08-12 Spain
Zar06-12 Spain
Tar06-12 Spain
SOS173
SOS148
SOS149
SOS151
SOS404
SOS468
GQ166866 MRCV USA 2001
CBAnd1Fig Spain 04-2012
EU871528 RCV-A1 Australia 2007
SOS492
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SOS140
CBMontemor14-1 Portugal 02-2014
SOS150
SOS155
CBEstremoz14-3 Portugal
CBMora14-1 Portugal
CBEstremoz14-1 Portugal 01-2014
CBCoruche14-1 Portugal
CBCoruche14-2 Portugal 02-2014
CBLavra10-13-1 Portugal 10-2013
CBEstoi13-7 Portugal 12-2013
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7-13 Barrancos Portugal 01-2013
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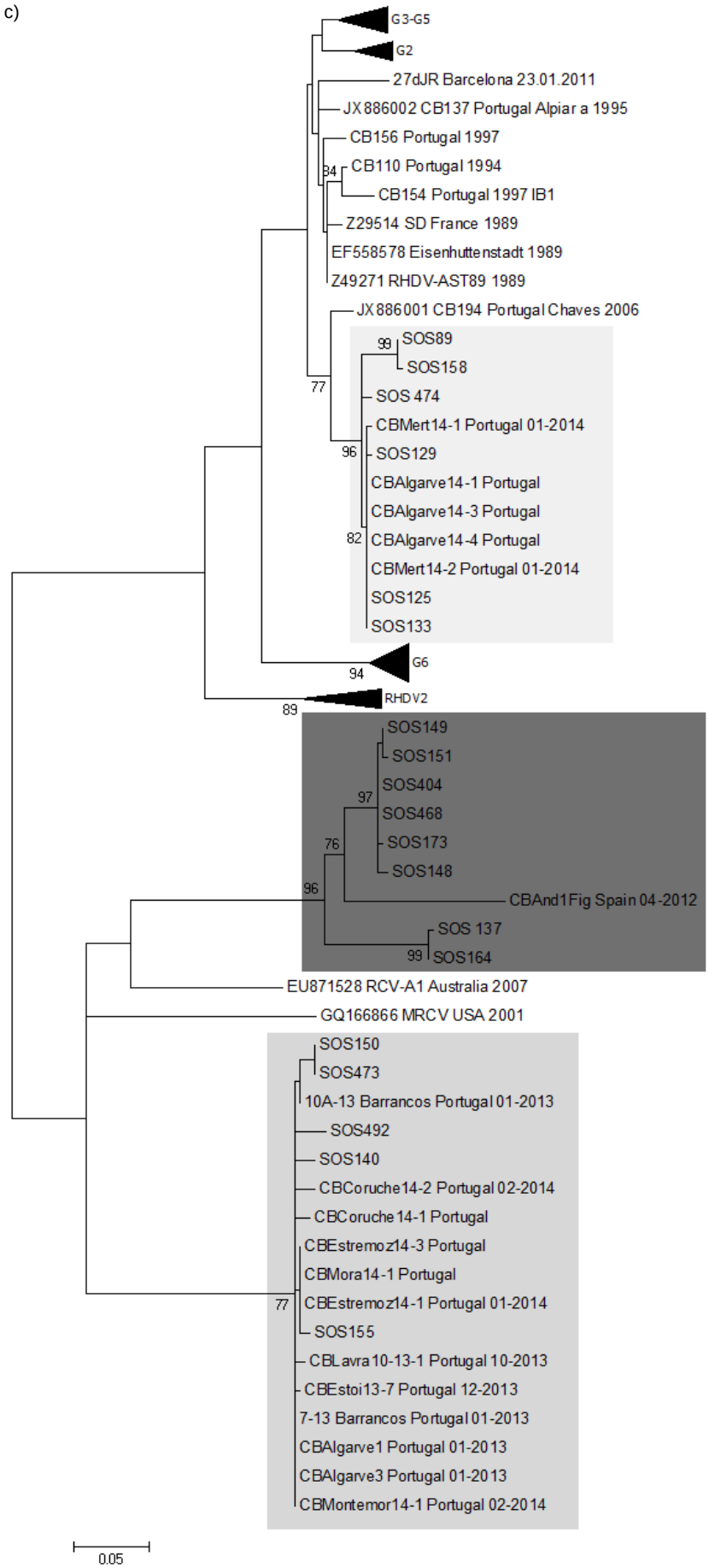
c)


G3-G5
G2
27dJR Barcelona 23.01.2011
JX886002 CB137 Portugal Alpiar a 1995
CB156 Portugal 1997
CB110 Portugal 1994
CB154 Portugal 1997 IB1
Z29514 SD France 1989
EF558578 Eisenhuttenstadt 1989
Z49271 RHDV-AST89 1989
JX886001 CB194 Portugal Chaves 2006
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CBMert14-1 Portugal 01-2014
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CBAlgarve14-1 Portugal
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CBAlgarve14-4 Portugal
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EU871528 RCV-A1 Australia 2007
GQ166866 MRCV USA 2001
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CBCoruche14-2 Portugal 02-2014
CBCoruche14-1 Portugal
CBEstremoz14-3 Portugal
CBMora14-1 Portugal
CBEstremoz14-1 Portugal 01-2014
SOS155
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7-13 Barrancos Portugal 01-2013
CBAlgarve1 Portugal 01-2013
CBAlgarve3 Portugal 01-2013
CBMontemor14-1 Portugal 02-2014
0.05

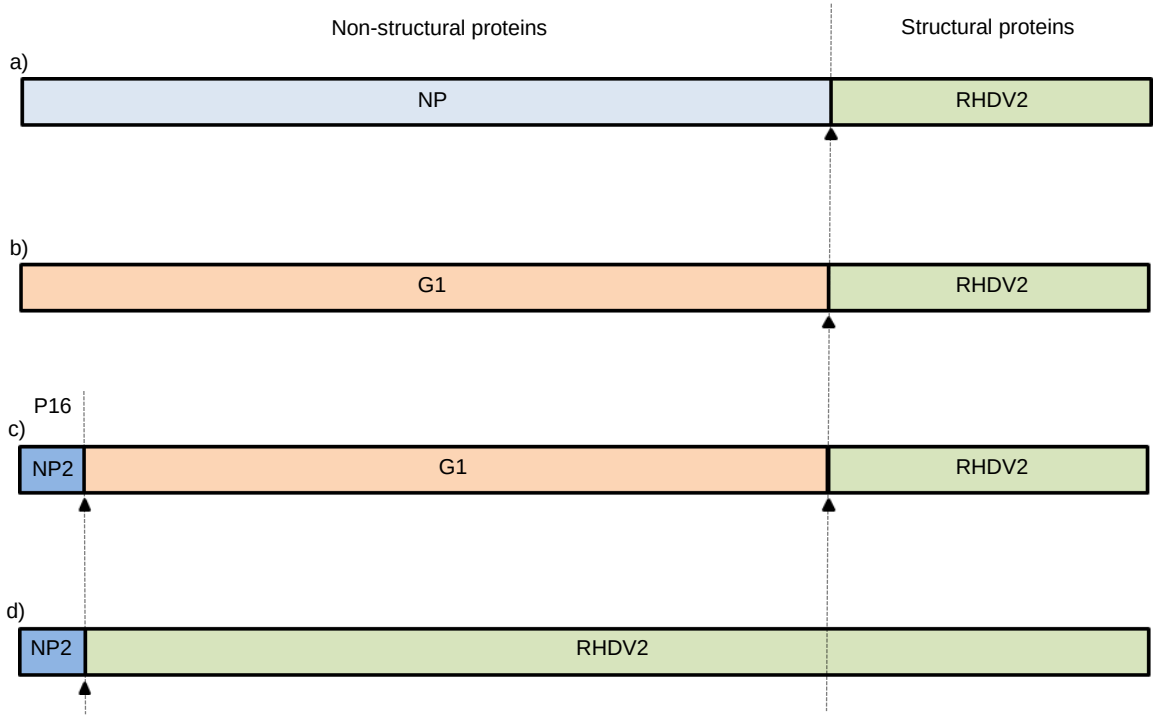

Fig. 6. Genomic composition of RHDV2 recombinants detected. a) NP/RHDV2 recombinant. b) G1/RHDV2 recombinant. c) NP2/G1/RHDV2 recombinant. d) NP2/RHDV2 recombinant. Arrowheads indicate the recombination breakpoint. NP: non-pathogenic lagovirus closely related to RCV-A1. NP2: non-pathogenic lagovirus closely related to CBAnd1.

The recombination events between RHDV2, G1 and non-pathogenic strains imply that co-circulation has occurred in the same geographical range and time frame. The high number of distinct circulating RHDV2 strains and widespread distribution over different localities and years confirms the viability and epidemiological success of these recombinant strains. Taking this into account, further monitoring should be performed on the high diversity of circulating RHDV strains. Most important, it is likely that there are at least two types of non-pathogenic strains circulating in the wild rabbit populations: one similar to the CBAnd1 strain and another closely related to Australian non-pathogenic strains. The isolation and characterization of these non-pathogenic strains may be one further step in understanding the emergence, pathogenicity and evolution of RHDV and, in particular, of RHDV2.

3.1.2. Amino acid polymorphisms

Percentage of polymorphisms varied for each protein and for each type of RHDV2 recombinant. Comparison of our G1/RHDV2 and NP/RHDV2 recombinants with the G1 and non-pathogenic lagoviruses for the non-structural proteins (except p16) revealed similar polymorphism diversity. This value was higher than that obtained for the comparison of our non-recombinant RHDV2 strains with publicly available RHDV2 non-recombinant strains. These results could be due to the relatively recent emergence of RHDV2 when compared to other strains (included G1 strains were sampled between 1989 and 2011, and non-pathogenic strains present already high levels of divergence between them) and consequently due to the low number of available RHDV2 sequences. The capsid is the protein with highest diversity (9%). This may be important on the evolution of RHDV2, as this is the most exposed protein and thus under higher pressure from the host immune system. Therefore, a higher genetic diversity might assist the maintenance and spread of this new variant by aiding the virus to escape such pressure.

In both G1 and non-pathogenic RHDV2 recombinant strains, the highest percentage of polymorphisms was observed for the p16 protein (13% and 16%, respectively). The remaining non-structural proteins varied between 3-8% for G1 and 3-9% for non-pathogenic strains. A similar result was observed for EBHSV strains [128]. Unfortunately, diversity for the p16 protein was not possible to be calculated for the RHDV2 group, as none of the sequences obtained in this study were non-recombinants at that protein (or “true” RHDV2 strains). A recent study revealed a high number of amino acid changes for p16 in Australian strains, and an association was made between mutations in this protein and virulence [61]. Indeed, these findings suggest that p16 could be undergoing a higher selective pressure. Although further studies are needed on the functional characterization of this non-structural protein, it may have a potential role on virulence or perhaps host-interaction, the main factors driving such selective pressure [129].

3.1.3. Selection analysis

In order to further understand the selective pressures that may be responsible for the mechanisms underlying the evolution and pathogenesis of RHDV2, selection analyses were performed to detect codons with signatures of positive selection. Positive

selection has previously been reported in the VP60 of classical RHDV strains [112,130] and in the Australian calicivirus [44].

Signatures of selection were detected with four methods available in Datamonkey software and in PAML [118,119]. The comparison between the pairs M1a vs. M2a and M7 vs. M8 was statistically significant (Table 2). Thus, models that allow for positive selection (M2a and M8) better fit the data and the BEB method implemented in PAML was used to detect codons under selection (p>95%).

An analysis of the sequences based on their non-structural subsets resulted in a lack of consensus between the methods used, where no positively selected sites were detected by at least three methods, most likely due to the low number of sequences available. On the other hand, analysis of RHDV2 capsid sequences detected codons 10 and 307 as being putatively under positive selection (five methods with P values <0.05 or posterior probability values >0.95; Table 3).

Table 2. Comparison between pairs of methods.

Comparison	2Δl [a]	d.f. [b]	Significance
M1a vs M2a	7.3	2	0.05
M7 vs M8	18.8	2	0.001

[a]Difference between twice the log likelihood values
[b]Number of degrees of freedom

Table 3. Results of the selection analysis.

Sites	Regions	Amino acids	Polarity	Charge	Methods (significance)				
					SLAC	FEL	MEME	FUBAR	PAML (M8)
10	A	Q V	Polar Nonpolar	Neutral Neutral	0.044*	0.013*	0.000*	0.995**	0.992**
307	C	S G N A	Polar Nonpolar Polar Nonpolar	Neutral Neutral Neutral Neutral	0.023*	0.002*	0.005*	0.999**	0.997**

*P value
**Posterior probability value

The codons identified belong to regions A and C, respectively, of the six main regions (A-F) identified in caliciviruses capsids [131]. According to the RHDV atomic model [36], codon 10 is located in the N-terminal arm which in RHDV, in contrast to other

caliciviruses, corresponds to a short fragment of the capsid [131], while codon 307 is located on the P2 subdomain. Codon 10 is a newly found positively selected codon, apparently exclusive of RHDV2 although it was not detected previously [73]. Since it is located in a non-exposed region of the capsid, it is difficult to determine the underlying selective pressures.

On the other hand, codon 307 had already been detected on other selection analyses, including in RCV-A1 [44,130]. This codon is located at a highly variable and exposed region. Although region E is thought to contain the main antigenic determinants, such as the recently reported binding site for histo-blood group antigens in RHDV2 [132], and which are thought to be important for infection, the high diversity and detection of a codon under positive selection in region C indicates that this region may also be important on host interaction and binding in RHDV2 [72,133,134]. Indeed, selective pressure seems to be mainly driven by the host immune response, and variability observed in region C suggests the existence of other antigenic determinants [130]. The maintenance of this positively selected codon among RHDV and RHDV2 sequences is of great interest, especially taking into account the antigenic differences found between them [64]. This indicates that, even though the new RHDV2 strain is quite antigenically dissimilar from RHDV, they still may share regions evolving under the same pressure, likely mediated by interactions with the host immune system, which might explain the existence of some antibody cross-reaction [64,132].

Furthermore, codon 307 is located at a putative N-glycosylation site [130]. In some viruses, glycosylation, which involves the attachment of a high mannose core to the amide nitrogen of an asparagine's residue, is used as a strategy to evade host immune response and to increase virulence [135]. Mutational studies on other viruses on the deletion of N-glycosylation sites have proved to reduce virulence and to enhance host immune response [136,137]. No evidence exists for the occurrence of N-glycosylation in RHDV (nor RHDV2). However, this putative N-glycosylation site that appears to be under positive selection in both RHDV and RHDV2 strains, is absent in non-pathogenic strains isolated in Europe [52,53], thus this codon may have impact on viral characteristics linked to transmissibility, host immune evasion or virulence. More information on positively selected sites may help to discover new potentially antigenic determinants and to link variation caused by selective pressure with possible changes in virulence and pathogenicity [129].

3.1.4. Haplotype network

In order to obtain a phylogeographic pattern of distribution for RHDV2, all publicly available RHDV2 capsid sequences, including the ones sequenced in this study, were used to construct a phylogenetic network (Figure 7).

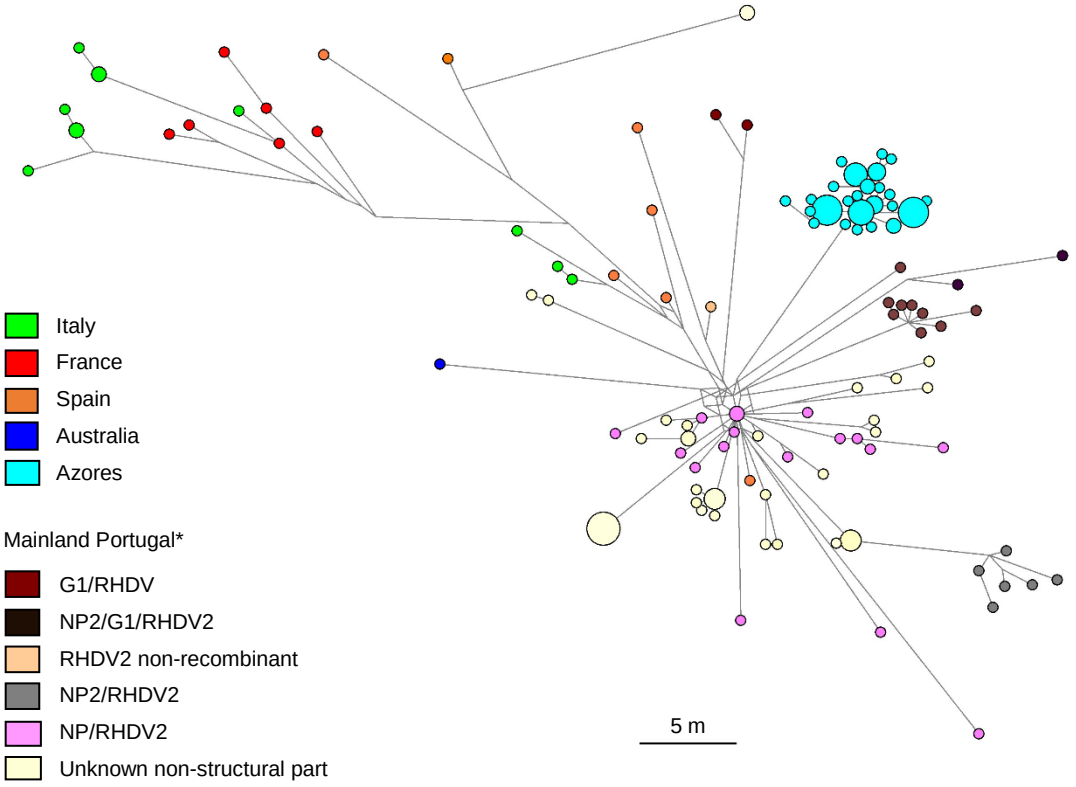

Fig. 7. Median-joining network of RHDV2 capsid sequences. Pie charts represent viral capsid haplotypes (nucleotide positions 5305-7378) and size is proportional to the frequency of each haplotype. Haplotypes are coloured according to the country of sampling, and in the case of mainland Portugal*, samples were further divided into the types of recombinants. Median vectors are omitted to facilitate visualization. Scale length corresponds to 5 mutations (m).

In this haplotype network, it is possible to distinguish a geographical pattern for RHDV2 strains. Although RHDV2 place of origin is uncertain, the first outbreaks were reported in France [62]. Assuming that emergence did occur there, it seems that RHDV2 dispersed into Italy, likely due to the proximity between these two countries. This is confirmed by the close clustering of French and Italian haplotypes. Then, the virus seems to have reached the Iberian Peninsula, though not clear if its origin is French or Italian as haplotypes from both countries appear related with Iberian haplotypes. Nevertheless,

several mutations separate the French/Italian haplotypes from the Iberian haplotypes. This might reflect the high substitution rates commonly observed for RNA viruses such as RHDV [1,138] or a lack of sampling of “intermediate” haplotypes.

Interestingly, for the Iberian haplotypes, NP/RHDV2 recombinants appear somewhat dispersed, while G1/RHDV2 and NP2/RHDV2 strains group closely together. This pattern, however, could reflect the lack of information on the non-structural backbone for several Iberian RHDV2 haplotypes. Indeed, recombinant strains sharing the same non-structural backbone probably originated from the same parental strains and thus their capsid haplotypes should be related as recombination is likely to have occurred only once and not several times.

The occurrence of haplotypes at high frequencies corresponds to samples collected in close localities, where the virus quickly spread between individuals before mutations could occur in the capsid gene.

Azorean RHDV2 haplotypes cluster together and are more closely related with G1/RHDV2 strains from the Iberian Peninsula. This is compatible with the scenario observed for other European countries experiencing RHDV2 where the virus rapidly spread and replaced existing strains [62,76]. Nevertheless, the geographical isolation between islands and the close relationship between haplotypes retrieved from the different islands suggest that spread of RHDV2 to some (if not all) of the Azorean islands might have been man-mediated [139].

The phylogenetic network also suggests that the first reported RHDV2 strain in Australia possibly originated from the Iberian Peninsula. This goes in accordance with phylogenetic analyses performed on this sample, which revealed to be a G1/RHDV2 recombinant at the non-structural/structural junction closely related to this type of recombinants that are unique to Iberia [70]. Nevertheless, this haplotype presents several mutations comparing to Iberian RHDV2 haplotypes.

Unfortunately, since it is not possible to account for and sequence all occurring epizootics, there may be missing information between distant haplotypes. Thus, to fully disclose the geographical patterns of RHDV2 distribution and follow its evolutionary history, broader monitoring and sampling is needed of occurring RHDV2 outbreaks across the world.

3.1.5. Substitution rate and tMRCA estimation

Substitution rate and tMRCA were estimated for all RHDV2 capsid sequences using a Bayesian MCMC approach. Mean substitution rates of 4.3 x 10^{-3} subs/site/years were estimated for RHDV2, with 95% highest posterior density intervals (HPD) ranging from 3.3 x 10^{-3} to 5.3 x 10^{-3} subs/site/year. This estimate, although characteristic of RNA viruses [138], is higher than the proposed for classical RHDV (mean 2.7 x 10^{-3} subs/site/year) [85]. This is expected as RHDV2 is a recent and worldwide dispersed epidemic. However, this result may also be an overestimation as transient non-advantageous, but not lethal, mutations have not yet been purged. Also, the high haplotypic diversity found in the network (Figure 7) may be related with these non-purged transient deleterious mutations. Interestingly, recent estimates revealed a higher mean substitution rate of 5.6 x 10^{-3} subs/site/year for the non-pathogenic Australian calicivirus RCV-A1, but likely due to the same effect [44]. Moreover, the great epidemiological success of RHDV2 may be responsible for the high substitution rate as expected for RNA viruses [140].

Based on the mean substitution rate, tMRCA was estimated at 9.6 years ago (7.5 – 12.1 years, 95% confidence interval). This places a shared common ancestor for RHDV2 around 2007, a few years prior to the first detection of this new variant in 2010 [62], suggesting that RHDV2 was probably already circulating prior to its detection. However, assuming that it may have emerged around 2007, it is possible that it did not yet possess the pathogenicity/virulence of the strains found in 2010. If so, it is important to assess the possible causes for the emergence and epidemiology of RHDV2, focusing on the frequent recombination events observed for this new variant, but also on its ability to cross the species boundaries [68,74,75]. Both for classical RHDV and RCV-A1, sample composition (structural vs. non-structural proteins) gives different estimates of tMRCA, being always higher for VP60 [44,85]. Thus, future work should focus on obtaining more sequences from the non-structural parts of the different RHDV2 recombinants in order to improve this estimation.

3.2. Characterization of old RHDV samples

3.2.1. Recombination, phylogenetic and genetic distances analyses

The four recombinant genomes (P16, P19, P30 and P95) obtained from archived samples collected in Portugal in the mid-1990s (see Annex V for samples information) were aligned with publicly available full-length RHDV genomes. The alignment was screened for recombination using RDP [116]. Seven methods detected these four strains as recombinants with strong statistical support (P values <0.001, Table 4). There was also consistent evidence for a single recombination breakpoint located near the RdRp/VP60 boundary (nucleotide positions 5242-5399; Table 4) splitting the genome into two distinct subsets, one corresponding to the non-structural coding region and the other to the structural coding region. RDP further identified G1 as the parental genome for the structural subset, but no significant similarity with any of the currently known genetic groups was found for the non-structural subset. This indicated that these four samples are recombinants and that their non-structural coding region had its origin in strains from a new genetic group.

Table 4. Results of the recombination analysis for old samples using RDP.

Strains	Most likely donor strain (Genogroup)		Breakpoint †	Methods (average P value)						
	NS*	S**		RDP	GENECONV	BootScan	MaxChi	Chimaera	SIScan	3Seq
P16 P19 P30 P95	Unknown (Unknown)	AST89 (G1)	5242-5399	8.63E-31	1.16E-19	2.78E-31	6.21E-12	2.85E-12	2.87E-17	1.42E-16

***Non-structural**
****Structural**
†95% confidence interval

To further confirm the recombination event and disclose the evolutionary relationships of this new genetic group, ML phylogenetic trees were constructed for the non-structural and the structural parts. For the non-structural part, P16, P19, P30 and P95 samples clustered apart from all groups, forming a highly supported monophyletic group (bootstrap value of 100; Figure 8a). This group fell between the G1-G6 and

RHDV2 clusters and seems to have shared a common ancestor with pathogenic strains rather than with non-pathogenic strains. In contrast, in the ML tree for the structural part, these strains were closely related with G1 strains from 1989, with strong statistical support (bootstrap value of 95; Figure 8b). Hence, the distinct positioning of the four strains in the ML phylogenetic trees suggests that they are recombinants and that recombination may have occurred between G1 (structural part) and a phylogenetically distinct genetic group that had never been reported and that composes the non-structural part.

Several recombination events had been reported in pathogenic RHDV strains, including in the recently described RHDV2 [42,43,90]. The old recombinant strains described in this study present the same modular pattern described for RHDV2 by combining structural and non-structural protein subsets with distinct origins [43]. However, for RHDV2, G1 was a donor for the non-structural backbone, while for these samples G1 acted as a donor for the structural backbone (VP60 and VP10). The consistency in the location of the recombination breakpoint observed for these recombination events supports that despite the identification of other breakpoints in the RHDV genome [42,90,91], the non-structural/structural junction is a recombination hotspot in lagoviruses [43] as observed for other caliciviruses [141-144].

The nucleotide distances estimated for both the non-structural and the structural genomic parts further supports that for the non-structural part the newly identified recombinant strains originated from a new RHDV genetic group. Indeed, in this fragment these strains diverged ~13% from the G1, G2, G3-G5 and G6 groups and from RHDV2, and ~22% from the non-pathogenic strains MRCV (GenBank accession number GQ166866) and RCV-A1 (data not shown), while distances between the different genogroups were lower, ranging between 6% and 12%. For the structural part, lower distances were observed between the recombinant strains and G1 strains (data not shown), confirming that a G1-like strain acted as a donor for this genomic fragment [46,48].

Host-pathogen co-evolution depends on the interplay between pathogen virulence, transmission potential and host resistance. Pathogens usually experience a trade-off between transmission and virulence and those with the optimum balance between these two factors are usually maintained [145]. It is possible that the recombination event affected the transmission and/or virulence of these strains, decreasing virus fitness. Since the structural proteins of the Portuguese recombinant strains are similar to G1 strains that were widespread in the Iberian Peninsula and were clearly pathogenic while the non-structural proteins originated from the new genetic group, it can be hypothesized

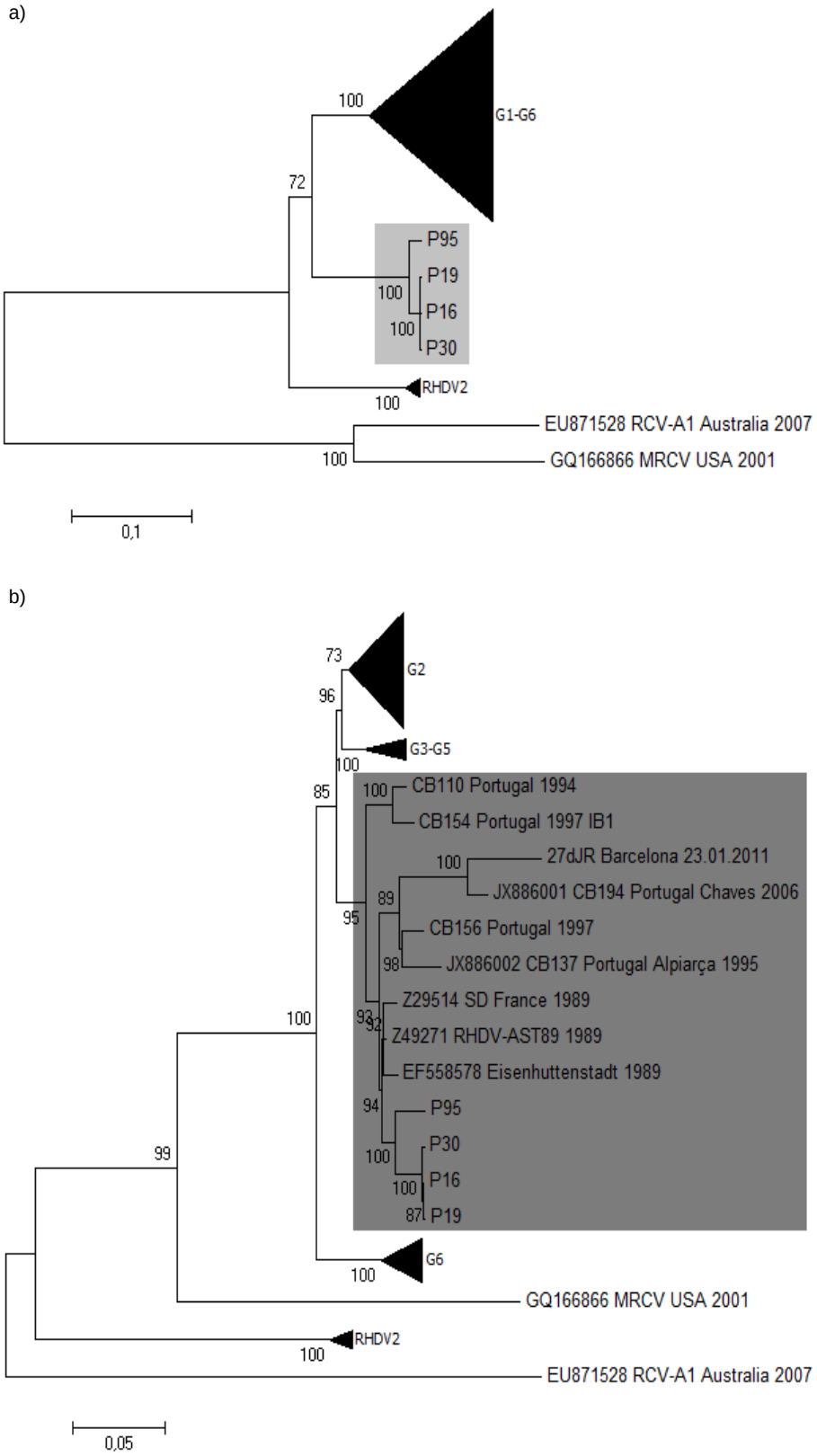

Fig. 8. ML phylogenetic trees for genome regions defined by the recombination analysis of the old recombinant samples. Genogroups containing the sequenced strains are shaded. The remaining genogroups are collapsed and indicated. Horizontal branch lengths are drawn to a scale of nucleotide substitutions per site and the tree is mid-point rooted. Only bootstrap values ≥70% are shown for key nodes. a) ML tree based on the non-structural proteins (nucleotides 10-5295). Old sequences appear in a new genogroup (shaded medium grey). b) ML tree based on capsid protein sequences (nucleotides 5296-7368). Sequences cluster with G1 strains (shaded dark grey).

that the characteristics of the non-structural fragment may have interfered with virulence and perhaps transmission. Moreover, it is possible that this new lineage already presented a lower fitness that was not restored with the “acquisition” of the genes encoding the structural proteins of G1. This is in contrast with RHDV2 recombinants that managed to spread and persist in the rabbit populations and replaced G1 in Portugal [73,76]. Alternatively, it is possible that the recombinant strains were outcompeted by more competitive strains. Indeed, these strains might had been replaced by G1 strains in wild rabbits or by G6 strains in domestic rabbits as these are commonly associated with the rabbit industry [2,10,146].

Thus, the recombinant strains reported here most likely went extinct, despite having a structural backbone of a successful genetic group such as G1 that circulated in the Iberian Peninsula for more than 20 years [46,48]. Notwithstanding, this recombination event was associated with the earliest record of a species jump in RHDV. In fact, P95 is an Iberian hare found dead in the field which at necropsy presented clinical signs of a lagovirus infection (see Annex V). The molecular analysis revealed that this hare was indeed infected by RHDV [147]. Thus, these recombinants had the ability to cross the species barrier, which was now also observed for RHDV2 [68,74], they did not sustain further infections, constituting a “dead-end” event.

The evolutionary forces driving recombination in RNA viruses are unclear, but recombination has been associated with major changes in virus evolution, emergence and epidemiology. For example, emergence of new pathogenic viruses, expansion of host range and modification of tissue tropism have been observed following recombination events [87]. Moreover, recombination is frequently considered a repairing mechanism for deleterious mutations and a mechanism for quickly generating diversity [89]. Even though the impact of recombination in RHDV is not yet completely understood, it is evident that gene exchange between lagoviruses occurred for classical strains [42,90,91] and is even more frequent for RHDV2 strains [43]. Therefore, it is crucial to understand more about the possible impacts of recombination, by characterizing each type of RHDV recombinant strain and by performing experimental infections to assess the virulence of each recombinant. If differences are found, it may be possible to determine which types of recombinants present an advantage that may allow them to take over other strains and pinpoint relevant mutations.

3.3. Identification and characterization of non-pathogenic strains in Iberian Peninsula

Despite the high number of samples analysed (n=187) and the screening efforts, it was not possible to detect viral sequences of non-pathogenic lagoviruses from Portuguese rabbit and hare samples. Even the more sensitive qRT-PCR method revealed unfruitful in detecting these non-pathogenic strains.

Non-pathogenic lagoviruses had been detected in Europe [52,53] and Australia [55], but never in the Iberian Peninsula. However, the recombination events detected in Iberian RHDV2 strains suggest that at least two types of non-pathogenic strains were circulating when RHDV2 arrived to the Iberian Peninsula. The inability to isolate such strains might be due to several factors, e.g., the non-pathogenic backbones of RHDV2 recombinants are the only remnants of elusive non-pathogenic lagoviruses.

It is also possible that, due to the high substitution rate observed for non-pathogenic strains [44], primers used are not able to hybridize with the target sequence. Even though two pairs of primers were used, a universal pair for lagoviruses and a specific pair for non-pathogenic sequences related to the RHDV2 recombinants, mutations in Iberian non-pathogenic strains may be preventing primer annealing [148].

Furthermore, screening was performed on wild adult rabbits hunted on the field rather than young ones (see Annex VII for samples information). As demonstrated in other studies, non-pathogenic lagoviruses seem to be more commonly found in young rabbits [53,55]. Thus, detecting an Iberian non-pathogenic strain may be more successful by directing the sampling at a larger number of young rabbits.

4. Concluding remarks

Recombination is central in creating genetic diversity in lagoviruses and seems associated with key events in their evolution. Indeed, the strain recovered from the first reported RHDV outbreak in China is the product of a recombination event [90]. Likewise, RHDV2 emergence was followed by the appearance of several recombinant strains in Iberia [43], and recombination seems to have become even more complex with new types of recombinants being found in this study. Thus, by focusing the analyses only on the capsid gene of lagoviruses, genetic diversity has been clearly underestimated. In fact, most RHDV2 sequences obtained in other studies are only of the capsid gene and there is a lack of information regarding the possible occurrence of recombination on these strains with genogroups other than G1 or other non-pathogenic lagoviruses. Hence, as this study demonstrates, to fully assess lagoviruses' evolution, characterization of strains should focus in obtaining complete genome sequences rather than targeting partial fragments of the genome.

Furthermore, it is necessary to understand the impact that these events may have on the evolution, transmissibility and pathogenicity of lagoviruses. Particularly, it urges to understand the function of the non-structural proteins and their role in virus virulence, tropism and host susceptibility, taking into account the mixed origins of the recombinant strains. However, this may be hampered by the lack of a suitable cell culture system for RHDV.

Another step in further understanding lagoviruses' evolution is the characterization of the extant non-pathogenic strains. Although elusive in Iberia, there is an increasing number of non-pathogenic samples being detected in other European countries and Australia [52-55], and they may have had an important role in the emergence of these viruses. Indeed, it is possible that pathogenic strains have evolved from non-pathogenic ones [60], and, if so, it is important to describe the genetic mechanisms involved in the emergence of pathogenicity.

With the need of better understanding the emergence, evolution and epidemiology of rabbit lagoviruses and their impact on rabbit populations, particularly in the Mediterranean ecosystems where rabbit is a keystone species, a higher effort on sampling and viral characterization should be employed.

5. Future perspectives

The diversity found in RHDV2 strains analysed for this study stresses the need for a continued monitoring of circulating strains on rabbit populations to characterize each type of strain and track down the evolution of rabbit lagoviruses. As there are at least four types of RHDV2 recombinants, it would be interesting to assess through experimental infections whether there are any differences in fitness for each recombinant form. With continued monitoring, it will be possible to determine which types of recombinants occur more often on rabbit populations, or even if new types of recombinants arise, and to continuously assess their impact on rabbit populations in the Iberian Peninsula.

The fact that most sequences obtained for RHDV and RHDV2 are of the capsid gene may be concealing genetic information. As it was demonstrated for Iberian strains, only full genome sequences will allow to correctly characterize each strain and have a complete picture of virus evolution. This study demonstrated that if the non-structural part had not been sequenced, the new recombination events detected would have gone unnoticed. Thus, it would be important to increase the number of complete genomes for each genetic group and detect possible recombination events between them, including for other countries in Europe and Australia.

Even though the RT-PCR method is commonly used for detecting and amplifying RHDV, and the genome walking strategy applied in this study proved to be a very efficient method for full genome amplification, a higher amount of data could be processed through the development of high-throughput sequencing methods [149,150] specific for lagoviruses. This would allow the amplification and sequencing of multiple full length genomes in a faster and cheaper way, and would greatly benefit the characterization of lagoviruses strains worldwide.

Similarly, detection and characterization of non-pathogenic lagoviruses could benefit from the development of a NGS approach. We were not able to detect any of these strains in the Iberian Peninsula, despite the evidence for their co-circulation with RHDV2. While it is possible, but highly unlikely, that these strains no longer exist in Iberia, other approaches may be performed for their detection. For example, designing new primers that amplify conserved regions of the non-structural proteins and focusing sampling efforts in juvenile rabbits (< 2 months old) [55].

6. References

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Annexes

Annex I - Protocol for collection of duodenum samples

To collect the duodenum:

1. In the abdomen, search for the stomach and for the stomach-intestine junction.

2. In the upper part of the junction, tie a string with caution to not disrupt the tissue.

3. Tie a second string 6cm apart from the other string.

4. Cut the portion between the two strings (the strings should remain in the animal).

5. Wash in the washing solution by holding the collected piece with a tweezers.

6. With a syringe, wash **gently** the lumen (internal side) of the sample by injecting washing solution 2-3x or until it comes out clean; alternatively, the collected piece should be washed in a bath of washing solution.

7. Put the sample in the tube with the storing solution (RNA later) and invert the tube a few times gently, making sure the solution covers the entire sample.

8. Store at -20ºC.

Notes:

- Samples should be collected as soon as possible after the death of the animal.
- Washing solution (PBS 1x) should be changed between animals to avoid contaminations.
- Material used to collect samples (tweezers, scissors, scalpels, etc) should be rinsed with bleach, ethanol (70%) and finally washing solution between each animal.

Annex II – PCR primers and conditions

			Forward primer 5' - 3'	Reverse primer 5' - 3'	Annealing temperature	Extension period
Lagoviruses screening	**General**	RHDV4831F + EBHSV_VP60_0467R	GTGTATGCCATGACTCCGAT	GCGTCGATGACAACATGAG	48º C	1 min
	RHDV2-specific	RHDV6186F + RHDV6748R	CATTGACCACGACAGAGGTAAC	CGTTAGTTGAACCGGCCTCAG	67º C	20 s
Non-pathogenic (NP) lagoviruses	**Universal**	Rab1b + Rab2	CAGCDSGCACTGCYACCACAGCATC	GAAKCKRAACTGCATGCCACCAGCCCA	65º C	20 s
	Non-pathogenic specific	RHDV0078F + RHDV0270R	CTTCCTGGACCTCAGGGACAAG	CATGTGGGTCCGAATTTGTGC	51º C	10 s
Full genome amplification	**General**	RHDV0001F + RHDV0201R	GTGAAAGTTATGGCGGCTATGTCG	GCCACATTTGTCACATGTCTCCAG	52º C	20 s
		RHDV0078F + RHDV1530R	CTTCCTGGACCTCAGGGACAAG	GTGGTCTCTGCTTCGTCATG	52º C	1 min 15 s
		RHDV1432F + RHDV2515R	AGGTGCACCCTGCCATCATACAA	ATGAGCGTGGTCTGTGAGTGGAA	52º C	1 min
		RHDV2356F + RHDV3592R	CAACATCTTTGGCGCATGGT	TACGCCAGCACGTCAATCTT	56-51º C	1 min 30 s
		RHDV3396F + RHDV5035R	GTACATATCTAACACCCACAC	GCCTRAGTATRCTGGACTTGTC	48º C	1 min 30 s
		RHDV2914F + RHDV4634R	GCAAACCACCTTGTCAACCT	ATGGCGTCAAGTATGGTCATGG	48º C	1 min 30 s
		RHDV4429F + RHb5400RC	GTTGGCGTTGACATGACATC	CTAGTAGTGGCCACAACACC	52º C	1 min
		RHDV5412F + RHDV6748R	CACTACTAGTGTGGTCACCAC	CGTTAGTTGAACCGGCCTCAG	50º C	1 min
		RHDV5609F + RHDV6778R	TGCTGAGCCAGATGTACGCT	AGTCCAATTGTCACTGGCAG	52º C	1 min 15 s
		RHDV6661F + RHDV7437R	GCCGCTCCTATTGGCAAGAAC	ATAGCTTACTTTAAACTATAAACCCAA	53º C	1 min
	G1	RHDV0078F + RHDV1503R	CTTCCTGGACCTCAGGGACAAG	TTCCCAAGGACCATGGTGTGT	53º C	1 min 15 s
		EBHSV3327F + RHb4650RC	CAATGGGAGCGGATGGATGATTC	GGTTTGCACAATCATGGC	52º C	1 min 10 s
		RHDV6662F + RHDV7437R	CACCTGTGGGTAAGAACACA	ATAGCTTACTTTAAACTATAAACCCAA	50º C	45 s
	RHDV2	RHDV2161F + RHDV3592R	GAGGGTGCYAACAAGTTCAAC	TACGCCAGCACGTCAATCTT	50º C	1 min 15 s
		RHb4170 + RHb5400RC	GAAGATCAACCATGTGAAAG	CTAGTAGTGGCCACAACACC	52º C	1 min
		RHb5000 + RHDV6335R	GTGTCAAAATTGGACAAGTCC	AAGGGCACGAACGACATGTCA	52º C	1 min
	NP	RHb750 + RHDV2421R	CTTGGTACACCAGACTTGG	GGTTYGTCATATTCACACAGCC	50º C	1 min 30 s
		RHDV5172F + RHDV6335R	CATTAAGCCAGAACTGGAGCGTC	AAGGGCACGAACGACATGTCA	53º C	1 min 15 s

Annex III – RHDV2 samples information

										Genogroup	
Laboratory code	**Year**	**Gender**	**Age**	**Weight (g)**	**Species**	**Type**	**Origin**	**Collection place**	**Lesions**	**Upstream VP60 (5')**	**VP60**
SOS_89	2014	M	A	-	*O. c. algirus*	Wild	Hunted	Ponte de Sôr	n/a	G1	RHDV2
SOS_125	2014	F	A	-	*O. c. algirus*	Wild	Found	Ferreira do Alentejo	n/a	G1	
SOS_129	2014	n/d	J	-	*O. c. algirus*	n/d	Found	Mértola	n/a	G1	
SOS_133	2014	n/d	A	-	*O. c. algirus*	n/d	Found	Mértola	n/a	G1	
SOS_137	2015	n/d	J	-	*O. c. algirus*	Wild	Found	Barrancos	n/a	G1*	
SOS_140	2015	n/d	J	-	*O. c. algirus*	Wild	Found	Mora	n/a	NP	
SOS_148	2015	F	A	1118	*O. c. algirus*	Wild	n/d	Mértola	Epistaxis; congested head tissues; congested neck tissues; haemorrhagic lungs.	RHDV2*	
SOS_149	2015	F	A	836	*O. c. algirus*	Wild	n/d	Mértola	n/a	RHDV2*	
SOS_150	2015	M	A	856	*O. c. algirus*	Wild	Found	Mértola	Epistaxis; blepharoconjunctivitis compatible with myxomatosis.	NP	
SOS_151	2015	M	A	858	*O. c. algirus*	Wild	n/d	Mértola	n/d	RHDV2*	
SOS_155	2015	F	A	1088	*O. c. algirus*	Wild	Found	Serpa	Epistaxis.	NP	
SOS_158	2015	F	J	390	*O. c. algirus*	Wild	Hunted	Ponte de Sôr	Epistaxis; congested neck tissues; haemorrhagic lungs and heart; trachea with foamy and bloody liquid; pale liver.	G1	
SOS_164	2015	F	J	428	*O. c. algirus*	Wild	Found	Serpa	Epistaxis; haemorrhagic lungs; pale and friable liver.	G1*	
SOS_173	2015	M	J	362	*O. c. algirus*	Wild	n/d	Mértola	Epistaxis; haemorrhagic lungs; trachea with foamy and bloody liquid; pale and friable liver.	RHDV2*	
SOS_404	2015	M	J	357	*O. c. algirus*	Wild	Found	Mértola	Epistaxis; haemorrhagic lungs; liver with light brown colour.	RHDV2*	
SOS_468	2015	F	J	486	*O. c. algirus*	Wild	Found	Mértola	Multifocal haemorrhages in the lungs; trachea with foamy liquid; abdominal cavity filled with bloody liquid; pale liver.	RHDV2*	
SOS_473	2015	M	J	654	*O. c. algirus*	Wild	Found	Mogadouro	Multifocal haemorrhages in the lungs; trachea with foamy liquid; abdominal cavity filled with bloody liquid; wrinkly and pale liver, with brown spots across the organ.	NP	
SOS_474	2015	F	J	883	*O. c. algirus*	Wild	Found	Mirandela	Multifocal haemorrhages in the lungs; abdominal cavity filled with bloody liquid; wrinkly and pale liver, with brown spots across the organ.	G1	
SOS_492	2016	n/d	J	-	*O. c. algirus*	Wild	Found	Estremoz	n/d	NP	

M – Male; F – Female; A – Adult; J – Juvenile; n/d: no data; *Recombinant at p16.

Annex IV – Dataset and accession numbers for RHDV2 analyses

Accession number	Genogroup	Analysis
EF558578	G1	1, 2
Z29514	G1	1, 2
Z49271	G1	1, 2
KP129400	G1	1, 2
KP090974	G1	1, 2
KP090975	G1	1, 2
JF438967	G1	1, 2
JX886001	G1	1, 2
JX886002	G1	1, 2
DQ189078	G2	1
AF295785	G2	1
EU003580	G2	1
EF558579	G2	1
EF558580	G2	1
EU003579	G2	1
M67473	G2	1
RHU54983	G2	1
EF558572	G3-G5	1
EF558573	G3-G5	1
EF558574	G3-G5	1
EF558585	G3-G5	1
EF558575	G3-G5	1
X87607	G3-G5	1
EF558577	G3-G5	1
DQ189077	G3-G5	1
EF363035	G3-G5	1
EF558576	G3-G5	1
AB300693	G6	1
AF258618	G6	1
AY523410	G6	1
DQ205345	G6	1
DQ280493	G6	1
EF558581	G6	1
EF558582	G6	1
EF558583	G6	1
EF558584	G6	1
EU003578	G6	1
EU003581	G6	1

Accession number	Genogroup	Analysis
EU003582	G6	1
HM623309	G6	1
JF412629	G6	1
KF677011	G6	1
AM268419	NP	1
EU871528	NP	1, 2
GQ166866	MRCV	1
KM115714	RHDV2	1, 2, 3
KM115715	RHDV2	1, 2, 3
KM115716	RHDV2	1, 2, 3
KM115712	RHDV2	1, 2, 3
KM115713	RHDV2	1, 2, 3
KF442964	RHDV2	1, 2, 3
KF442963	RHDV2	1, 2, 3
KF442961	RHDV2	1, 2, 3
KF442962	RHDV2	1, 2, 3
KM115697	RHDV2	1, 2, 3
KM115698	RHDV2	1, 2, 3
KM115680	RHDV2	1, 2, 3
KM115681	RHDV2	1, 2, 3
KM115682	RHDV2	1, 2, 3
KM115711	RHDV2	1, 2, 3
KM115689	RHDV2	1, 2, 3
KM115683	RHDV2	1, 2, 3
HE800531	RHDV2	1, 2, 3
HE800532	RHDV2	1, 2, 3
KM979445	RHDV2	1, 2, 3
KP129399	RHDV2	1, 2, 3
KP129398	RHDV2	1, 2, 3
KP129395	RHDV2	1, 2, 3
KP129396	RHDV2	1, 2, 3
KP129397	RHDV2	1, 2, 3
KP090976	RHDV2	1, 2, 3
KM878681	RHDV2	3
FR819781	RHDV2	3
HE800530	RHDV2	3
HE819400	RHDV2	3
HE800529.1	RHDV2	3

Accession number	Genogroup	Analysis
KT280060.1	RHDV2	3
KJ683901.1	RHDV2	3
KJ683899.1	RHDV2	3
KJ683898.1	RHDV2	3
KJ683897.1	RHDV2	3
KJ683895.1	RHDV2	3
KJ683896.1	RHDV2	3
KM115710.1	RHDV2	3
KM115709.1	RHDV2	3
KM115708.1	RHDV2	3
KM115707.1	RHDV2	3
KM115706.1	RHDV2	3
KM115705.1	RHDV2	3
KM115704.1	RHDV2	3
KM115703.1	RHDV2	3
KM115702.1	RHDV2	3
KM115701.1	RHDV2	3
KM115700.1	RHDV2	3
KM115699.1	RHDV2	3
KM115696.1	RHDV2	3
KM115695.1	RHDV2	3
KM115694.1	RHDV2	3
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KM115692.1	RHDV2	3
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KM115687.1	RHDV2	3
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KM115685.1	RHDV2	3
KM115684.1	RHDV2	3
KM115679.1	RHDV2	3
KM115678.1	RHDV2	3
KM115677.1	RHDV2	3
KM115676.1	RHDV2	3
KM115675.1	RHDV2	3
KM115674.1	RHDV2	3
KM115673.1	RHDV2	3

1 – Recombination and phylogenetic analyses; 2 – Amino acid polymorphisms analysis;
3 – Selection analysis, haplotype network and substitution rate and tMRCA estimation

Accession number	Genogroup	Analysis
KM115672.1	RHDV2	3
KM115671.1	RHDV2	3
KM115670.1	RHDV2	3
KM115669.1	RHDV2	3
KM115668.1	RHDV2	3
KM115667.1	RHDV2	3
KC741409.2	RHDV2	3
KJ957809.1	RHDV2	3
KJ957810.1	RHDV2	3
JQ929052	RHDV2	3
JX106022.1	RHDV2	3
JX106023.1	RHDV2	3
KC345611.1	RHDV2	3
KC345612.1	RHDV2	3
KC345613.1	RHDV2	3
KC907712.1	RHDV2	3
KT000290	RHDV2	3
KT000291	RHDV2	3
KT000292	RHDV2	3
KT000293	RHDV2	3
KT000294	RHDV2	3
KT000295	RHDV2	3
KT000296	RHDV2	3
KT000297	RHDV2	3
KT000298	RHDV2	3
KT000299	RHDV2	3
KT000300	RHDV2	3
KT000301	RHDV2	3
KT000302	RHDV2	3
KT000303	RHDV2	3
KT000304	RHDV2	3
KT000305	RHDV2	3
KT000306	RHDV2	3
KT000307	RHDV2	3
KT000308	RHDV2	3
KT000309	RHDV2	3
KT000310	RHDV2	3
KT000311	RHDV2	3

Accession number	Genogroup	Analysis
KT000312	RHDV2	3
KT000313	RHDV2	3
KT000314	RHDV2	3
KT000315	RHDV2	3
KT000316	RHDV2	3
KT000317	RHDV2	3
KT000318	RHDV2	3
KT000319	RHDV2	3
KT000320	RHDV2	3
KT000321	RHDV2	3
KT000322	RHDV2	3
KT000323	RHDV2	3
KT000324	RHDV2	3
KT000325	RHDV2	3
KT000326	RHDV2	3
KT000327	RHDV2	3
KT000328	RHDV2	3
KT000329	RHDV2	3
KT000330	RHDV2	3
KT000331	RHDV2	3
KT000332	RHDV2	3
KT000333	RHDV2	3
KT000334	RHDV2	3
KT000335	RHDV2	3
KT000336	RHDV2	3
KT000337	RHDV2	3
KT000338	RHDV2	3
KT000339	RHDV2	3
KT000340	RHDV2	3
KT000341	RHDV2	3
KT000342	RHDV2	3
KT000343	RHDV2	3

1 – Recombination and phylogenetic analyses; 2 – Amino acid polymorphisms analysis;
3 – Selection analysis, haplotype network and substitution rate and tMRCA estimation

Annex V – Old samples information

Laboratory code	Year	Gender	Age	Weight (g)	Species	Type	Collection place	Lesions
P16	1994	M	A	1661	*O. cuniculus*	Dom	Porto	Epistaxis; haemorrhagic trachea; congested lungs, with haemorrhages and edema; slightly congested liver; slightly congested kidney with deep red coloration; abdominal cavity with non-coagulated blood; heart in diastole.
P19	1994	F	A	2460	*O. cuniculus*	Dom	Porto	Epistaxis; haemorrhagic trachea; congested lung, with haemorrhages and edema; slightly congested kidney with deep red coloration; slightly congested spleen; heart in diastole with atria filled with blood; liver with a distinct lobular pattern.
P30	1994	M	A	2622	*O. cuniculus*	Dom	Porto	Congested lung, with haemorrhages and edema.
P95*	1996	n/d	n/d	n/d	*L. granatensis*	Wild	Torres Novas	Haemorrhagic trachea; congested lung, with haemorrhages and edema; congested liver; dilated blood vessels filled with blood; abdominal cavity with blood.

M – Male; F – Female; A – Adult; Dom – Domestic; n/d: no data. *[147]

Annex VI – Dataset and accession numbers for old samples analyses

Accession number	Genogroup
EF558578	G1
Z29514	G1
Z49271	G1
KP129400	G1
KP090974	G1
KP090975	G1
JF438967	G1
JX886001	G1
JX886002	G1
DQ189078	G2
EU003579	G2
EU003580	G2
KP144789	G2
KP144790	G2
KT006721	G2
KT006722	G2
KT006723	G2
KT006724	G2
KT006725	G2
KT006726	G2
KT006727	G2
KT006728	G2
KT006729	G2
KT006730	G2
KT006731	G2
KT006732	G2

Accession number	Genogroup
KT006733	G2
KT006734	G2
KT006735	G2
KT006736	G2
KT006737	G2
KT006738	G2
KT006739	G2
KT006740	G2
KT006741	G2
KT006742	G2
KT006743	G2
KT006744	G2
KT006745	G2
KT006746	G2
KT006747	G2
KT280058	G2
KT280059	G2
KT344770	G2
KT344771	G2
KT344772	G2
KT344773	G2
KT344774	G2
DQ189077	G3-G5
EF363035	G3-G5
EF558575	G3-G5
EF558576	G3-G5

Accession number	Genogroup
EF558577	G3-G5
KP144792	G3-G5
X87607	G3-G5
AB300693	G6
AF258618	G6
AY523410	G6
DQ205345	G6
DQ280493	G6
EF558581	G6
EF558582	G6
EF558583	G6
EF558584	G6
EU003578	G6
EU003581	G6
EU003582	G6
HM623309	G6
JF412629	G6
KF677011	G6
GQ166866	MRCV
EU871528	NP
KM979445	RHDV2
KP129399	RHDV2
KP129398	RHDV2
KP129395	RHDV2
KP129396	RHDV2
KP129397	RHDV2
KM878681	RHDV2

Annex VII – Duodenum samples information

Laboratory code	Year	Gender	Age	Species	Type	Origin	Location
SOS_214	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_215	2015	M	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_216	2015	M	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_217	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_218	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_219	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_220	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_221	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_222	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_223	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_224	2015	M	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_225	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_226	2015	M	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_227	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_228	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_229	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_230	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_231	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_232	2015	F	J	*O. c. algirus*	Wild	Hunted	Serpa
SOS_233	2015	M	J	*O. c. algirus*	Wild	Hunted	Serpa
SOS_234	2015	F	J	*O. c. algirus*	Wild	Hunted	Serpa
SOS_235	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_236	2015	F	J	*O. c. algirus*	Wild	Hunted	Serpa
SOS_237	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_238	2015	M	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_239	2015	M	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_240	2015	M	J	*O. c. algirus*	Wild	Hunted	Serpa
SOS_241	2015	M	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_242	2015	F	J	*O. c. algirus*	Wild	Hunted	Serpa
SOS_243	2015	M	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_244	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_245	2015	F	J	*O. c. algirus*	Wild	Hunted	Serpa
SOS_246	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_247	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_248	2015	M	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_249	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_250	2015	F	J	*O. c. algirus*	Wild	Hunted	Serpa
SOS_251	2015	M	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_252	2015	M	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_253	2015	F	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_254	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola

SOS_255	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_256	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_257	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_258	2015	M	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_259	2015	F	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_260	2015	F	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_261	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_262	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_263	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_264	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_265	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_266	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_267	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_268	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_269	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_270	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_271	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_272	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_273	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_274	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_275	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_276	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_277	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_278	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_279	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_280	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_281	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_282	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_283	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_284	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_285	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_286	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_287	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_288	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_289	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_290	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_291	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_292	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_293	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_294	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_295	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_296	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_297	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_298	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_299	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_300	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola

SOS_301	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_302	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_303	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_304	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_305	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_306	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_307	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_308	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_309	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_310	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_311	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_312	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_313	2015	M	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_314	2015	n/d	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_315	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_316	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_317	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_318	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_319	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_320	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_321	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_322	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_323	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_324	2015	M	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_325	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_326	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_327	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_328	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_329	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_330	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_331	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_332	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_333	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_334	2015	n/d	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_335	2015	M	A	*O. c. algirus*	Wild	Hunted	Castro Verde
SOS_336	2015	F	A	*O. c. algirus*	Wild	Hunted	Castro Verde
SOS_337	2015	F	A	*O. c. algirus*	Wild	Hunted	Castro Verde
SOS_338	2015	M	A	*O. c. algirus*	Wild	Hunted	Castro Verde
SOS_339	2015	F	A	*O. c. algirus*	Wild	Hunted	Castro Verde
SOS_340	2015	M	A	*O. c. algirus*	Wild	Hunted	Castro Verde
SOS_341	2015	F	A	*O. c. algirus*	Wild	Hunted	Castro Verde
SOS_342	2015	F	A	*O. c. algirus*	Wild	Hunted	Castro Verde
SOS_343	2015	M	A	*L. granatensis*	Wild	Hunted	Castro Verde
SOS_344	2015	F	A	*L. granatensis*	Wild	Hunted	Castro Verde
SOS_345	2015	F	A	*L. granatensis*	Wild	Hunted	Castro Verde

SOS_346	2015	F	n/d	*O. c. algirus*	Wild	Hunted	Castro Verde
SOS_347	2015	F	A	*L. granatensis*	Wild	Hunted	Castro Verde
SOS_348	2015	F	A	*O. c. algirus*	Wild	Hunted	Castro Verde
SOS_349	2015	F	A	*L. granatensis*	Wild	Hunted	Mértola
SOS_350	2015	F	A	*L. granatensis*	Wild	Hunted	Mértola
SOS_351	2015	M	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_352	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_353	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_354	2015	M	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_355	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_356	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_357	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_358	2015	F	A	*L. granatensis*	Wild	Hunted	Mértola
SOS_359	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_360	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_361	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_362	2015	F	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_363	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_364	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_365	2015	F	A	*L. granatensis*	Wild	Hunted	Mértola
SOS_366	2015	M	A	*L. granatensis*	Wild	Hunted	Mértola
SOS_367	2015	M	A	*L. granatensis*	Wild	Hunted	Mértola
SOS_368	2015	M	A	*L. granatensis*	Wild	Hunted	Mértola
SOS_369	2015	M	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_370	2015	F	A	*L. granatensis*	Wild	Hunted	Mértola
SOS_371	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_372	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_373	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_374	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_375	2015	M	J	*O. c. algirus*	Wild	Hunted	Mértola
SOS_376	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_377	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_378	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_379	2015	M	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_380	2015	M	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_381	2015	M	J	*O. c. algirus*	Wild	Hunted	Serpa
SOS_382	2015	n/d	n/d	*O. c. algirus*	Wild	Hunted	Serpa
SOS_383	2015	M	A	*L. granatensis*	Wild	Hunted	Serpa
SOS_384	2015	F	A	*O. c. algirus*	Wild	Hunted	Serpa
SOS_385	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_386	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_387	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_388	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_389	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_390	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_391	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola

SOS_392	2015	F	n/d	*O. c. algirus*	Wild	Hunted	Mértola
SOS_393	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_394	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_395	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_396	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_397	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_398	2015	M	A	*O. c. algirus*	Wild	Hunted	Mértola
SOS_399	2015	F	A	*L. granatensis*	Wild	Hunted	Mértola
SOS_400	2015	F	A	*O. c. algirus*	Wild	Hunted	Mértola

M – Male; F – Female; A – Adult; J – Juvenile; n/d: no data.